2014

The Effect of Damaged Bases on The End Joining of DNA Double Strand Break Ends

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THE EFFECT OF DAMAGED BASES ON THE END JOINING OF DNA DOUBLE STRAND BREAK ENDS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

at Virginia Commonwealth University

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July, 2014

ACKNOWLEDGEMENT

Firstly, all thanks to Allah, The Most Gracious, Most Merciful, for all the blessings that I received and continue to receive and for the strength that he gave me to complete this research.

Secondly, I offer my sincere gratitude to my advisor, Dr. Lawrence Povirk, who has supported me throughout my thesis with a lot of patience. I attribute my Masters degree to his encouragement and effort and without whom this thesis, would not have been completed. One simply could not wish for a better or friendly advisor. Dr. Povirk is a great, respectful and intelligent scientist. I am so lucky to be one of his students. For everything you've done for me, Dr. Povirk, I thank you. I would also like to thank the members of my committee, Dr. David A. Gewirtz and Dr. Michael A. McVoy for giving me valuable suggestions, and guidance throughout my thesis work.

I would like to thank all of my lab members for their consistent help and support, Dr. Konstantin Akopiants, Dr. Tong Zhou, Sri Lakshmi Chalasani and Ajinkya Kawale. Special thanks to Sri Lakshmi for all the great time we have had, and all support that she gave especially staying with me late night while am trying to finish my lab or thesis work.

I owe special thanks and gratitude to those who have supported me during this journey to obtain my Master degree, for without their presence in my life reaching my destination would have been more difficult.

- I start by thanking and expressing gratitude to the greatest treasure in my life, my parents: My father Dr. Abdullah Bafail and My Mother Mrs. Saffia Samarkandi; for their love, support, prayers, encouragement and for believing in me all the way. Thanks to you mom for always being by my side giving me strength. You always had my back. I pray GOD to give you and dad a healthy and peaceful life ahead.

- My dear husband, Ahmad, you stood by me and you have given me your valuable time and spent a year just for me to take care of me and our two sweet kids while am busy with my studies. Many thanks to you Ahmad for all things you have provided me. Thanks to my mother in law, Mrs. Noor Hantoosh for all the prayers and for raising such a wonderful man like Ahmad.

Also I would like to thank my two little Kids, Elyana and Abdullah, for making my hard studies tolerable with their beautiful smiles.

Last but not least, I would like to thank the Ministry of Higher Education, Saudi Arabia for the scholarship and their financial support.

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AP	apurinic/apyrimidinic
APE1	apurinic/apyrimidinic endonuclease
ATP	adenosine triphosphate
BER	base excision repair
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	deoxythymidine
ddTTP	dideoxythymidine
DSBs	double-strand breaks (in DNA)
EndoIII	Endonuclease III
Hr	hour(s)
HRR	homlogous recombination repair
IR	ionizing radiation
µl	microliter
NHEJ	non-homologous end joining

SCID	severe combined immune-deficiency
sec	seconds
SDS	sodium dodecyl sulfate
SSBs	single-strand breaks (in DNA)
ssDNA	single-strand DNA
TEB	buffer solution mixture of Tris base, boric acid and EDTA.
Tg1	Thymine glycol terminally located at double strand breaks
Tg2	Thymine glycol located at the second position of double strand breaks
Tg3	Thymine glycol located at the third position of double strand breaks
V(D)J	variable, diversity, joining
XLF	XRCC4-like factor
X4L4	XRCC4 -DNA ligase IV complex
XRCC	X-ray cross-complementation grou

Abstract

THE EFFECT OF DAMAGED BASES ON THE LIGATION OF DNA DOUBLE STRAND BREAK ENDS

By Duaa Bafail, Pharm.D.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2014

Advisor: Lawrence F. Povirk, Ph.D, Professor, Department of Pharmacology and Toxicology

DNA double strand breaks (DSBs) are extremely toxic to cells because they can lead to genomic rearrangements and even cell death. Two main pathways can repair DSBs: the homologous recombination repair (HRR) pathway and the non-homologous end-joining (NHEJ) pathway. NHEJ is the primary repair pathway in mammalian cells. HRR repairs single strand breaks (SSBs) or DSBs, mostly during late S phase and G2 phase of the cell cycle, by using an undamaged copy of the DNA sequence, and is therefore largely error-free, while the NHEJ pathway repairs DSBs without the requirement for sequence homology, may be error-free or error-prone, and is most active during G1 phase.

Thymine glycol (Tg), the most common oxidation product of thymine. It produced endogenously as a consequence of aerobic metabolism or via exogenous factors such as ionizing radiation (IR), it is one of the predominant types of base modifications produced by ionizing radiation. Due to clustering of radiation – induced ionizations, Many DSBs induced by ionizing radiation bear damaged bases, including (Tg) moieties at or near the DSB ends that may interfere with subsequent gap filling and ligation.

Artemis is a nuclease that is involved in the processing of termini during repair of DSBs and in modifying termini of complex DSBs. It has 5′–3′ exonuclease activity specific for single-stranded DNA, but, in the presence of DNA-PK, Artemis demonstrates endonuclease activity that is utilized in the removal of 3′ phosphoglycolate termini and 5′ overhangs, in the shortening of 3′ overhangs at DSBs, and in the opening of hairpin ends.

To assess the ability of NHEJ to rejoin DSBs accompanied by Tg lesions and to elucidate the aspects of the possible role of Artemis in DSB repair, linearized plasmids with Tg either at the 3' terminus of a blunt end (designated Tg1) or three or two bases from the end (Tg3 and Tg2, respectively), were subjected to a repair assay using XRCC4-like factor (XLF) deficient cell extracts, with or without the addition of XLF and/or Artemis, EndoIII and ddTTP. The data indicated that, the cell extract could ligate the plasmids with Tg1 and Tg2 with extremely low efficiency but could repair plasmid with Tg3 as efficiently as unmodified plasmid. In addition, Plasmids with Tg1and Tg2 were treated with Endonuclease III and ddTTP to test whether the end joining occurred before or after Tg removing, neither one had any effects on plasmids with Tg1. However, plasmids with Tg2 showed reduced in the intensity upon treatment with Endonucleases III and ddTTP, which suggested some ligation occur while Tg still present. In Artemis reaction, substrate with Tg2 and Tg3 could stimulate Artemis mediated trimming but not

Tg1. Addition of EndoIII or ddTTP to plasmid with Tg3 resulted in a significant decrease in the intensities of the bandsrepresenting ligated products compared to XLF alone, suggesting that in some of the ligated products Tg are still present, while in others Tg had been removed and replaced by polymerization with normal nucleotides.

Taken together, our results indicated that cell extract could ligate the plasmid with Tg located at the third base to DSB with high efficiency compared to plasmids with Tg1 and Tg2 which apparently this ability was severely inhibited when it located at or in the second position to DSB ends. Moreover, Artemis is also capable of trimming of thymine glycol at the second or third position from DSB ends with limited capability but inhibited by the presence of thymine glycol at the break site.

I. INTRODUCTION

Cancer remains a leading cause of death globally. Indeed, in 2008, the World Health Organization (WHO) estimated that the age-standardized rate of mortality caused by cancer, for adults aged 30 to 70, was 150 per 100,000 (World Health Statistics, 2013). Surgery, immunotherapy, chemotherapy, and radiation therapy are the four major methods of cancer treatment. While these are often used in combination, radiation therapy continues to be the preferred method of therapy employed by most oncologists. Radiation therapy contributes towards 40% of curative treatments for cancer (Baskar et al. 2012), and at least 50% of all cancer patients will receive radiotherapy at some stage during the course of their illness (Tobias 1992). The target of radiation therapy is DNA. By further damaging the DNA of cancer cells that have already accumulated mutations, ionizing radiation induces the death of those cells. Although radiation therapy is directed at the tumor, it is inevitable that the normal tissues surrounding the tumor will also be affected by radiation damage (Burnet et al. 1996); radiation is also a proven carcinogen, and if radiation is misdirected at neighboring non-tumor cells, it can cause DNA damage in previously normal cells. The robustness of the various cellular DNA damage repair responses is critically important for restoring normal cellular function and preventing erstwhile normal cells from becoming precancerous.

1.1 DNA structure

DNA, or deoxyribonucleic acid, is a double helix, generally right-handed in orientation, consisting of two strands, running in opposite directions (anti-parallel). Each strand is made of deoxyribose sugars connected to each other via phosphate groups. Specifically, the 3' carbon of each sugar molecule is joined through a phosphate group to the 5' carbon of the following sugar. This series of covalent bonds is called a 3'-5' phosphodiester linkage. Each deoxyribose is also connected, via a glycosidic bond involving the 1' carbon, to one of four bases: adenine (A), cytosine(C), guanine (G) and thymine (T). Bases on opposite strands of DNA are paired such that adenine is always opposite to thymine and guanine is always opposite to cytosine. The A-T base-pair has 2 hydrogen bonds and the G-C base-pair has 3 hydrogen bonds, making the G-C interaction about 30% stronger than the A-T interaction. (Yakovchuk et al. 2006).

1.2 DNA double-strand breaks

Some of the most lethal manifestations of DNA damage are DNA double strand breaks (DSBs), which can be induced by a variety of endogenous and exogenous sources. Endogenous DSBs can arise from reactive oxygen species attacking both DNA strands, resulting in two or more single strand breaks (SSBs) in close proximity (Greinert et al. 2012). V(D)J recombination — recombination of variable (V), diversity (D), and joining (J) gene segments — can also induce DSBs during endonuclease processing (Gellert 2002), and formation of DSBs is a normal part of homologous recombination that occurs during meiosis (Baudat et al 2013; Borde and de Massy 2013). Endogenous DSBs can arise from replication fork stalling, when a cell attempts to replicate past a nick or other damage in the leading DNA strand, or from fork collapse (Lehmann

and Fuchs 2006; Garinis et al. 2005), and may even occur from physical stress on the DNA molecules, arising from the activities of helicases and topoisomerases during replication. Exogenous DSBs can be caused by certain chemotherapeutic agents such as bleomycin and etoposide, and by ionizing radiation (IR) (Shrivastav et al. 2008).

1.3 Complex lesions

Double-strand breaks in DNA can be complicated by the addition of a wide range of end modifications or by various types of base damage occurring near the initial break; as the number of such lesions increases, the potential of the DSB to cause a mutation rate increases (Hada and Georgakilas 2008; Ward 1995). In particular, IR can induce clusters of oxidative damage in DNA, involving the phosphate-ribose backbone, the nucleotides bases, or both, which can lead to the occurrence of DSBs with damaged bases at or near the break termini (Sage and Harrison 2011; Shikazono et al. 2009). Different modifications of DNA ends at DSBs can include alteration of thymine into thymine glycol, ring fragmentation, and formation of 3′-phosphoglycolates, 5′-hydroxyl groups, or abasic sites. The presence of additional damage of this kind at the site of DNA double-strand breaks negatively affects the rate and accuracy of DNA break repair, and the ability of the cell to repair DNA damage will decrease as the structural complexity at the break site increases (Shikazono et al. 2009).

1.4 Thymine glycol

Thymine glycol (Tg), the most common oxidation product of thymine, is produced as a consequence of oxidative stress from cellular processes, such as intracellular respiratory metabolism (Adelman et al. 1988), or by genotoxic agents such as ionizing radiation (Frenkel et al. 1981; Wallace 2002). Approximately 10–20% of all genomic damage induced by ionizing radiation, including from IR used in cancer therapy, results in DNA fragmentation and oxidation of thymine, especially the conversion of thymine to thymine glycol (Frenkel et al. 1981).

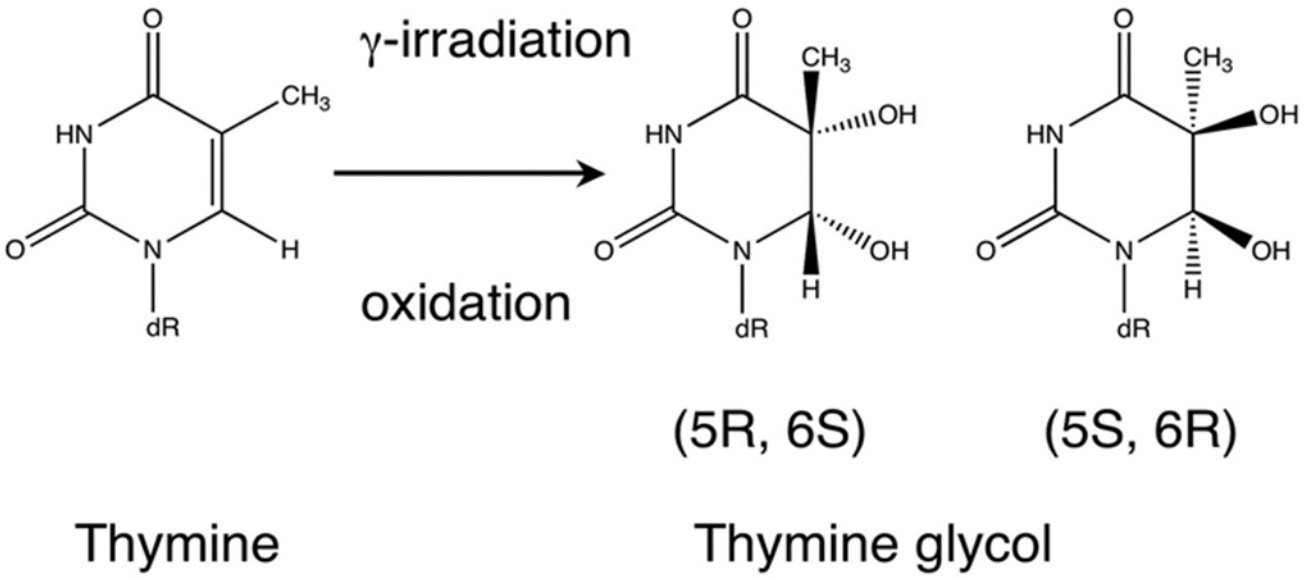


Figure 1-1. Formation of thymine glycol. Reactive oxygen species (ROS), such as peroxide and hydroxyl radicals, are generated as byproducts during normal oxidative metabolism, as well as by ionizing radiation. These can modify DNA bases. A common product of thymine oxidation is thymine glycol. Figure copied from Aller et al. 2007.

1.4.1 Oxidation of thymine into thymine glycol

Thymine glycol is one of the most commonly oxidized bases produced by ionizing radiation (Frenkel et al. 1981; Teoule 1987), partly because thymine is the most easily oxidized base (Dianov et al. 2000). Radiation generates oxidants that are thought to react with thymine and drive the formation of thymine glycol, thymine peroxide, thymine hydroperoxide, and other oxidized forms of the base; for example, thymine can be directly oxidized by hydroxy radicals to form Tg (von Sonntag and Schuchmann 1990; Cadet et al. 1986; Cathcart et al. 1984). Of note, the fragmentation of thymine is enhanced when the DNA is complexed with protein, presumably since the thymine in DNA is a sink for electrons ejected from proteins by IR. (von Sonntag and Schuchmann 1990; Cadet et al. 1986; Cathcart et al. 1984).

1.4.2 The effect of thymine glycol on DNA structure

Thymine glycol produces a distortion in the DNA duplex molecule; specifically, the addition of a hydroxyl group on position 5 causes the methyl group to take on a pseudo-axial orientation that results in distortion of the base pairs 5′ to the thymine glycol (Miller et al.,1994; Miaskiewicz et al.1995). In fact, primer extension studies with several DNA polymerases, using a template containing thymine glycol, show that extension occurs up to the lesion site, with insertion of adenine opposite the thymine glycol, but no further (Ide et al. 1985; Rouet and Essigmann 1985; Clark and Beardsley 1986; Hayes and LeClerc 1986). Thymine glycol has been known to block both the repair and replicative polymerases in vitro, and, if not repaired, can be lethal in vivo, in the absence of translation synthesis or recombination (Wallace 2002). There are, however, several sequence contexts where thymine glycol is bypassed by certain DNA

polymerases in vitro (Hayes and LeClerc 1986) and in vivo (Basu et al. 1989), and since Tg, like unmodified thymine, pairs with adenine, it is a poor premutagenic lesion (Hayes et al. 1988). Thymine glycol is, for the most part, removed via the single nucleotide patch base excision repair pathway (Dianov et al. 2000), but, if a thymine adjacent to a DSB is converted to Tg, a more robust cell response to this damage may be required.

1.5 Cellular Responses to DNA damage

Depending on when DNA damage occurs, the cell activates a checkpoint, which stops the cell cycle, in order to repair the damage before continuing DNA replication. In addition to an intra-S phase checkpoint, there are two major DNA damage checkpoints, which occur at the G1/S and G2/M boundaries of the cell cycle (Sancar et al. 2004; Houtgraaf et al. 2006). The two master regulators of these checkpoints are kinases called Ataxia Telangiectasia Mutated (ATM) and ATM- and RAD3-related (ATR). ATR primarily responds to damaged or stalled replication forks, whereas ATM is activated by the presence of DNA damage, including DSBs, as well as by disruption in chromatin structure; ATM can also activate the ATR pathway (Lee and Paull 2007; Bakkenist and Kastan MB 2003; Foray et al. 2003; Smith et al. 2010; Cimprich and Cortez 2008; Shechter et al. 2004). When these two enzymes are activated, they phosphorylate downstream proteins as the first step of a signal transduction pathway that leads to cell cycle arrest (Roos and Kaina 2013; Foray et al. 2003; Khanna et al. 2001; Shiloh 2001). The cell then initiates one of the DNA repair pathways. In the case of DNA double-strand breaks, there are two major repair pathways in mammalian cells: homologous recombination repair (HR repair or HRR) and non-

homologous end joining (NHEJ) (Valerie and Povirk 2003 ; Lieber 2010; Lieber et al. 2008). Once DNA DSB damage is recognized, the cell must choose which repair pathway to utilize.

1.6 Choice of DSB repair pathway

The choice between the HRR and NHEJ pathways depends on several factors, including the stage of the cell cycle and the severity and type of the DNA damage (Aylon et al. 2004; Aylon and Kupiec 2004; Huertas et al. 2008). HRR (also known as homology directed repair) is a pathway that requires a second, intact DNA duplex as a template, in order to repair a DSB; while the cell can use a sister chromosome as a template, in order to effect the most accurate replacement of damaged DNA, the cell usually uses sister chromatids. Hence, the cell uses HRR primarily during the S and G2 phases of the cell cycle, while DNA replication is ongoing, or after it has completed (Stark and Jasin 2003; Rothkamm et al. 2003). During DNA replication, HRR is believed to be preferred to NHEJ (Haber 1999a, 1999b, 2000); however, in general, NHEJ appears to be the dominant repair pathway used in mammalian cells, and it is active throughout the cell cycle, particularly in G0 or G1 phase (Rothkamm et al. 2003). Indeed, NHEJ is the preferred pathway for the repair of DSB in G1 phase, when sister chromatids are not available (Lieber 2010).

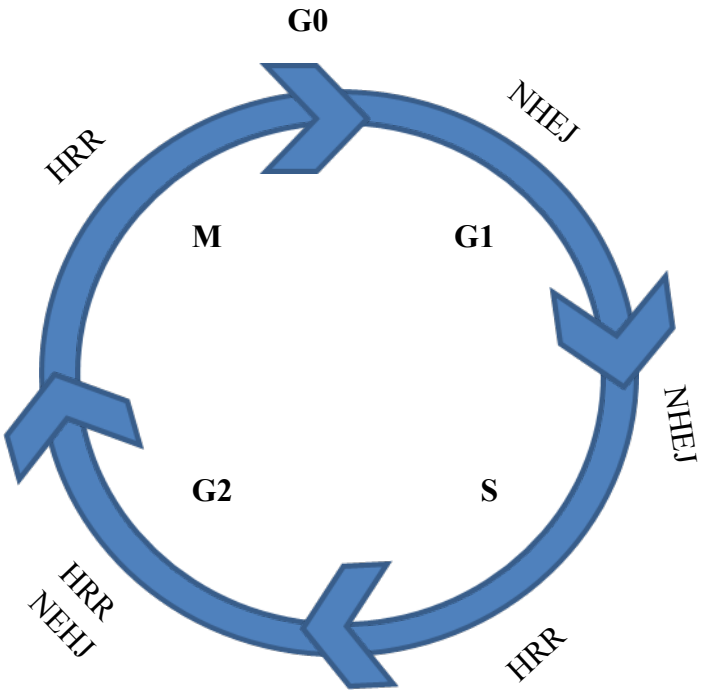


Figure 1-2. Cell Cycle Dependence of DNA Repair. Non-homologous end joining (NHEJ) is active throughout the cell cycle, while homologous recombination repair (HRR) requires a homologous DNA template in the form of sister chromatids, which are present only in S and G2 phases, sister chromosomes, or, in extreme cases, regions of microhomology, such as repeated DNA sequences.

1.7 Non-homologous End Joining (NHEJ) Repair

NHEJ, the predominant pathway to repair DSB in eukaryotic cells, is also the simplest, since it involves rejoining the two DNA ends by direct ligation (after processing by endonucleases, exonucleases, or polymerases), and thus, does not require lengthy homologous sequences to perform the repair. NHEJ is mainly active during the G0 and G1 phases, but maintains some activity throughout the cell cycle (Shrivastav et al. 2008). NHEJ proceeds through several stages: (1) DNA termini recognition, (2) formation of a synaptic complex, (3) DNA end processing, and (4) DNA ligation. Various proteins play a role in this process, including the Ku complex, which is composed of a Ku70/Ku80 heterodimer, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), XRCC4 (the protein product of the X-ray cross-complementation group 4 gene), DNA ligase IV, Artemis, and XLF (XRCC4-like factor) (Bassing and Alt 2004; Meek et al. 2004). Two components of the MRN complex, MRE11 and RAD50, also play a role in NHEJ (Paull and Gellert 1998).

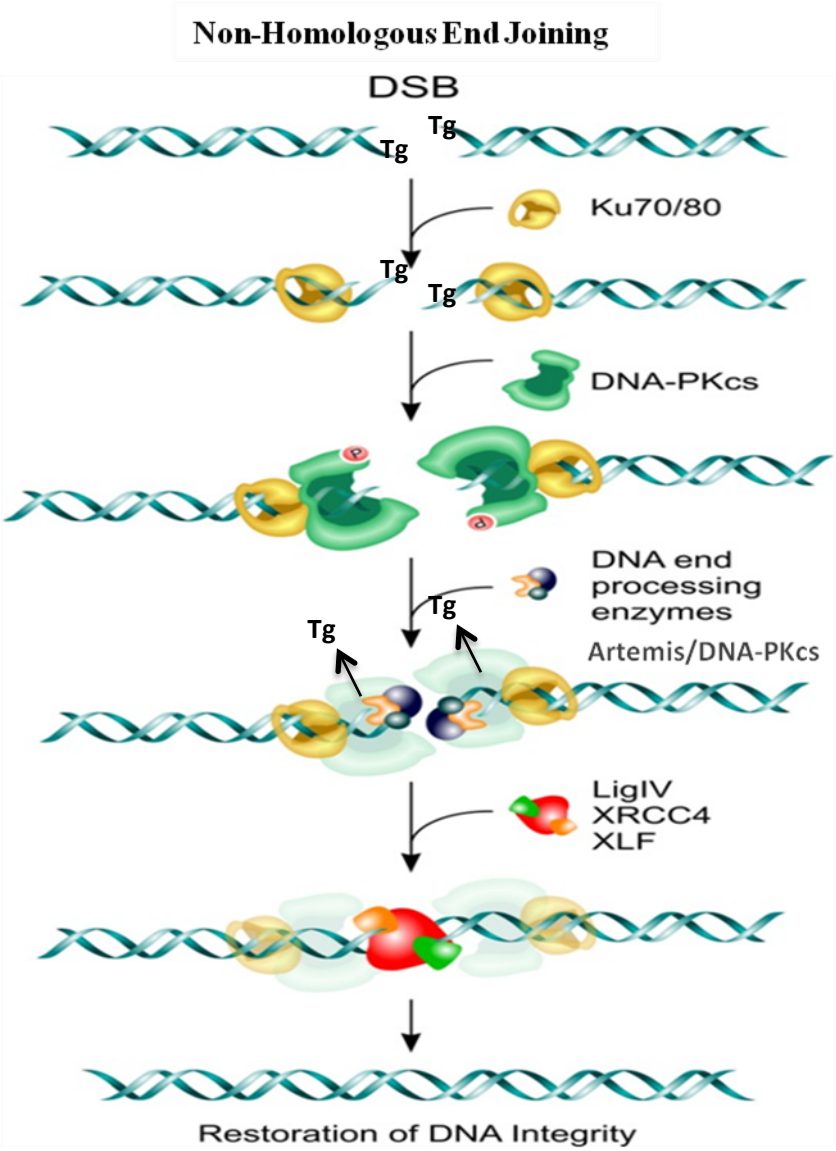


Figure 1-3. Non-homologous end joining repair pathway. The Artemis/DNA-PKcs complex is required for generating ligatable DNA ends in non-homologous end-joining (NHEJ) when complex damage is present. Ionizing radiation and radiomimetic agents induce double strand breaks (DSBs) with unligatable ends, such as 3′ phosphoglycolate termini. After the Ku70/Ku80 complex binds to DSB ends, the Artemis/DNA-PKcs complex generates ligatable DNA ends with minimal loss of nucleotides. Finally, the DNA ligase IV/XRCC4/XLF complex ligates the DSB ends. When NHEJ proteins leave the ligated DNA, NHEJ is completed.

1.8 Structural and biochemical properties of core NHEJ proteins

1.8.1 Ku

Ku was initially discovered as an autoantigen protein and found to be extremely abundant in the cell (Mimori et al. 1986). While Ku is a heterodimer made up of Ku70 and Ku80 subunits that bind preferentially to DNA end breaks at DSBs (Mimori and Hardin 1986; Gu Y. et al. 1997), these two subunits can also, when bound to DNA, form a heterotrimeric complex with the 465 kDa DNA-PKcs protein, forming an approximately 610 kDa complex on the DNA. Deficiency of either subunit leads to increased radiosensitivity in cells (Gu Y. et al. 1997; Smider et al. 1994; Taccioli et al. 1994; Rathmell and Chu 1994). The Ku complex is responsible for coordinating the activity of DNA-PK, and of various nucleases, at the DNA termini during NHEJ (Singleton et al. 1999; Weterings et al. 2009). The Ku proteins also interact with XRCC4 and DNA Ligase IV (Costantini et al. 2007; Mari et al. 2006). The two Ku subunits are similar in their structure, and each contains regions that contribute to the overall DNA binding domain of the heterodimer (Gell and Jackson 1999). In addition, the two proteins contribute to the overall function of the complex via individualized domains. Specifically, the C-terminal region of Ku80 forms a long flexible arm that may interact with domains in other proteins (Harris et al. 2004; Zhang Z. et al. 2004; Gell and Jackson 1999). Similarly, Ku70 contains an acidic N-terminal domain that is phosphorylated in vitro by DNA-PKcs (Chan et al. 1999) and a C-terminal domain with putative chromatin or DNA binding capability (Lees Miller and Meek 2003). In fact, of the two subunits, Ku70 may play a slightly larger role in DNA binding (Zhang and Yaneva 1992; Chou et al. 1992; Wang et al. 1994).

Binding of Ku to the DSB ends may assist in holding the broken ends together (Cary et al. 1997) and preventing the two strands from unraveling (Walker et al. 2001). At the same time, Ku can function as a helicase (Tuteja and Tuteja 2000) and may help unwind DNA at stalled replication forks. High resolution structural analysis of Ku shows a ring-like shape that does not change in conformation after binding to DNA (Walker et al. 2001), although some conformational changes have been indicated by biochemical studies (Lehman et al. 2008). The ring structure of the Ku70/80 DNA binding core encircles the DNA by interacting with the major and minor grooves of the double helix, and not with the nucleotide bases or with specific types of DNA ends (Walker et al. 2001; Paillard and Strauss 1991). This is consistent with the finding that, in vitro, Ku binds to ends of double-stranded DNA (dsDNA) with high affinity, but without any apparent sequence specificity (Downs and Jackson 2004; Zhang and Yaneva 1992; Mimori and Hardin 1986). It also provides the means by which Ku is able to perform a major function: to slide inward along the DNA strands, and thereby provide access to the extreme termini of the DSB to DNA repair proteins, such as DNA-PKcs (Downs and Jackson 2004; Yoo and Dynan 1999; Paillard and Strauss 1991).

1.8.2 DNA-PKcs

The catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs, is a large (over 4,000 amino acid) polypeptide that belongs to the phosphatidylinositol 3-kinase-related kinases (PIKKs) family of serine/threonine protein kinases (Lees-Miller and Meek 2003; Abraham 2004). In humans, the DNA-PKcs protein has a molecular weight of 469 kDa (Hartley et al. 1995). Cells that lack DNA-PKcs are radiosensitive, and have defects in the processing of coding

joints during V(D)J recombination. In addition, in mice, dogs, and horses, DNA-PKcs deficiency is associated with severe combined immunodeficiency (SCID) (Meek et al. 2008; Meek et al. 2004). DNA-PKcs and Ku interact via their respective C-terminal regions, and this interaction is required for the NHEJ related function of DNA-PKcs (Falck et al. 2005; Singleton et al. 1999); however, Ku and DNA-PKcs can only interact in the presence of DNA (Suwa et al. 1994). This suggests that, even though in vitro DNA-PKcs can bind to DNA independently of Ku (Yaneva et al. 1997), the binding of DNA-PKcs to sites of DNA damage in vivo is dependent on the presence of Ku (Uematsu et al. 2007).

As Ku translocates on the DNA in a direction inwards from the DSB, DNA-PKcs gains access to the extreme termini of the DNA at the DSB (Yoo and Dynan 1999; Paillard and Strauss 1991). This permits two molecules of DNA-PKcs, one on each end of the DSB, to interact across the break in a so-called "synaptic complex" (DeFazio et al. 2002). The interaction brings together the two DNA ends and stimulates the kinase activity of the two DNA-PKcs molecules; each DNA-PKcs molecule phosphorylates the other, leads to activate the kinase activity of DNA-PKcs which promote phosphorylation downstream target proteins needed for completion of the pathway (DeFazio et al. 2002; Meek et al. 2007). Once the DNA-PKcs-Ku-DSB complex is assembled at the DNA ends, it serves as a tether, holding the ends of the DSB securely together and protecting the DNA ends from attack by nucleases other than those required for NHEJ. This DNA-PKcs-Ku-DNA complex is referred to as DNA-PK, and the weak serine/threonine kinase activity of DNA-PKcs is greatly increased at dsDNA ends in the presence of Ku (Gottlieb and Jackson 1993). The protein kinase activity of DNA-PKcs is required for NHEJ (Kurimasa et al. 1999; Kienker et al. 2000); inhibitors of DNA-PK kinase activity make cells sensitive to IR by

interfering with DSB repair, so inhibiting DNA-PKcs function might serve as a mechanism by which to kill cancer cells (O'Connor et al. 2007; Zhao et al. 2006).

1.8.3 XRCC4 and DNA Ligase IV

In situations where complex or clustered DNA damage has occurred, such that DSBs are accompanied by damaged base, adducts, or abasic sites, the DNA ends need to be processed by nucleases, or filled in by polymerases μ and λ (Mahajan et al. 2002 ; Pawelczak et al. 2011), in order to clear out the non-DSB damage prior to ligation. Once the DNA ends are processed and damaged bases are removed or replaced, the ends must be ligated together in order to complete the repair of the DNA. XRCC4 is a homodimer with no known enzymatic activity (Mahaney et al. 2009), yet XRCC4 both stabilizes and stimulates the activity of DNA ligase IV (Grawunder et al. 1998, Grawunder et al. 1997). DNA ligase IV binds XRCC4 to form a highly stable complex (Sibanda et al. 2001; Modesti et al. 2003), and this complex, often referred to by the abbreviation X4L4, mediates the ligation of DNA ends (Mahaney et al. 2009). Interaction between XRCC4 and the XRCC4-like factor (XLF) stimulates the X4L4 complex and promotes functional flexibility to allow the ligation of seemingly incompatible ends, but even without XLF the X4L4 complex can ligate both compatible ends and certain incompatible overhangs (Gu J. et al. 2007a; Gu J. et al. 2007b; Lieber et al. 1997). Furthermore, DNA ligase IV displays the unusual property of being able to ligate one DNA strand at a time, so that, while DNA ligase IV is joining one already-processed strand, nucleases or polymerases can process the termini of the opposite strand (Gu J. et al. 2007b).

1.8.4 XLF

XRCC4-like factor (XLF) is structurally similar to XRCC4, and interacts with XRCC4 in vitro (Ahnesorg et al. 2006; Li et al. 2008). XLF, also called Cernunnos, can stimulate the activity of DNA ligase IV, especially in regards to the ligation of incompatible ends, which suggests that, under certain conditions, it might regulate the activity of the X4L4 complex (Gu J. et al. 2007b; Li et al. 2008; Lu et al. 2007). In fact, there is a marked increase in the ligation efficiency of imperfectly matched ends in the presence of XLF (Tsai et al. 2007; Lieber et al. 2008; Gu J. et al., 2007b). XLF is also required in NHEJ, as well as in V(D)J recombination (Ahnesorg et al. 2006). XLF is recruited to the DSB by Ku and through interaction with Ku-bound DNA (Yano et al. 2008), and is stabilized at the break upon interaction with the X4L4 complex (Mahaney et al. 2009).

1.9 Artemis

Artemis is a nuclease that is involved in the processing of termini during the repair of DSBs, and evidence suggests that Artemis is involved in modifying termini of complex DSBs (Covo et al. 2009). On its own, Artemis exhibits 5′–3′ exonuclease activity specific for single-stranded DNA; however, in the presence of DNA-PKcs and ATP, Artemis acquires endonuclease activity that is utilized in the removal of 3′-phosphoglycolate (3′-PG) termini, in order to provide 3′-hydroxyl termini suitable for patching and ligation (Povirk et al. 2007). This DNA-PKcs-dependent endonuclease activity is also utilized in the removal of 5′ overhangs, in the shortening of 3′ overhangs at DSBs, and in the opening of hairpins formed as intermediates during V(D)J recombination (Ma et al. 2002; Ma et al. 2005a; Goodarzi et al. 2006; Povirk et al. 2007). The

endonucleolytic trimming of DNA blunt ends with either 3′-PG or 3′-hydroxyl termini is accompanied by trimming of 5′ termini (Yannone et al. 2008).

Loss of function of Artemis, due to mutations in the corresponding gene, DCLRE1C, results in radiation-sensitive severe combined immunodeficiency (RS-SCID; OMIM entry 602450), as well as the Athabascan subtype of SCID, in humans (Moshous et al. 2001; Li et al. 2002 ; Moshous et al. 2003). Similar to cells lacking DNA-PKcs, cells lacking Artemis accumulate unopened DNA hairpins while undergoing V(D)J recombination (Rooney et al. 2002; Moshous et al. 2001). Artemis-deficient cells are sensitive to IR, but do not have major defects in DSB repair, suggesting that, in vivo, Artemis is only required for the repair of specific types of DNA damage (Poinsignon et al. 2004; Wang et al. 2005).

It has been suggested that Artemis' endonuclease activity requires phosphorylation by DNA-PKcs (Ma et al. 2002; Niewolik et al. 2006). However, mutation of Artemis at amino acids normally phosphorylated by DNA-PKcs has been shown to have no effect on its endonuclease activity in vitro, and does not affect its in vivo function in DSB repair or V(D)J recombination (Ma et al. 2005b; Goodarzi et al. 2006). Instead, Artemis endonuclease activity seems to require autophosphorylation of DNA-PKcs, which appears to increase access of Artemis to its DNA substrates (Goodarzi et al. 2006; Yannone et al. 2008).

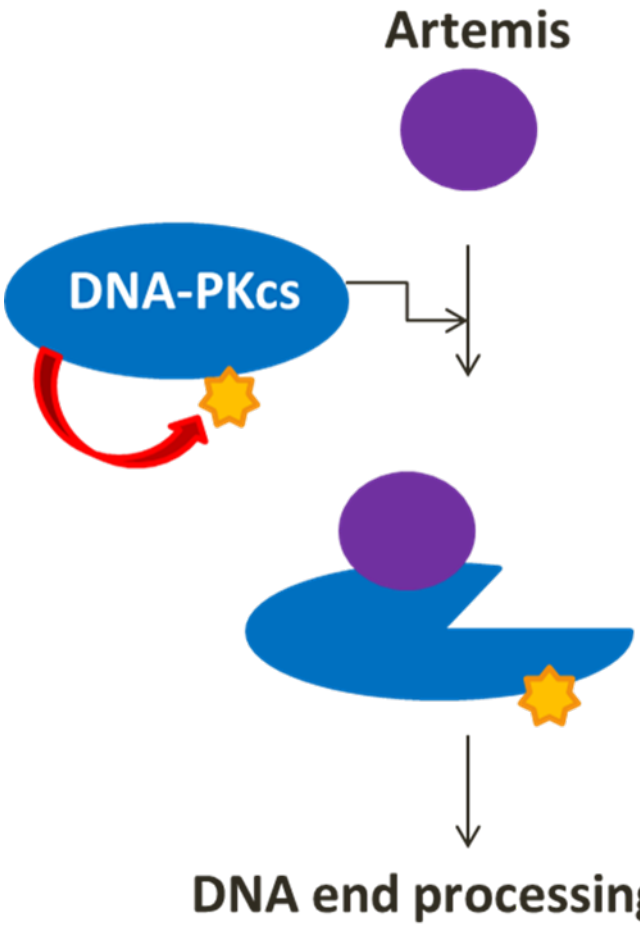


Figure 1-4. Regulation of Artemis in Non-homologous end joining (NHEJ) by DNA-PKcs. Autophosphorylation causes a conformational change in DNA-PKcs, resulting in an activated form of Artemis. The activated form of Artemis can create DNA ends suitable for ligation by DNA ligase IV.

1.10 Human EndoIII like protein (NTH) and bacterial Endonuclease III

EndoIII like protein (NTH) is one of the major DNA glycosylase that has role in base excision repair pathway (BER) as first step in the detection of the pyrimidines damage base and remove it by N-glycosylase activity (Lindahl and Wood 1999). One of this damage bases is thymine glycol (Ikeda et al., 1998). However, many reports documented that in the case of oxidative bases located at the 3'terminal position of a SSB, that activity of NTH1 is greatly reduced (Ali et al., 2005; Parsons et al., 2005). There are many genes that encode enzymes that are involved in the repair of damaged bases that are similar in many species ranging from bacteria to human (Ikeda et al., 1998)

Endonuclease III (Nth) is a protein from *E. coli* that acts as both an N-glycosylase and an AP-lyase. The N-glycosylase activity releases damaged pyrimidines from double-stranded DNA, generating an abasic site (an apyrimidinic or AP site). The AP-lyase activity of the enzyme cleaves 3′ to the AP site, leaving a 5′ phosphate and a 3′ ring-opened sugar. Some of the damaged bases recognized and removed by Endonuclease III include urea, thymine glycol, and uracil glycol (Dizdaroglu et al., 1993; Hatahet et al., 1994).

1.11 Specific Aims

NHEJ is necessary for repair of DSB that arise from different exogenous and endogenous sources, and it plays an important role in cellular resistance to theses breaks. However, one of the the primary obstacles to resolution of such breaks is the fact that biologically relevant sources of DNA damage produce DSBs that have complex end structures, that can interfere with the

ligation step of NHEJ. For example, breaks induced by radiation–mediated ionization are associated with cluster damages (Ward 1994; Goodhead 1994). NEHJ can repair the complex ends using a series of core of proteins, which include Ku70 and 80, DNA-PKcs and XRCC4-ligaseIV with XLF protein that recognize the ends, align paired ends together, and facilitate their ligation. Also, NHEJ can be considered as high or low fidelity and this depends on the degree of helix distortion in DNA. Low fidelity can actually be considered an advantage for NHEJ as it can in principle join complex ends together without the need for first processing the ends to remove damage. However, there are limits to what NHEJ can tolerate (Gu et al.2007; Chen et al.2001). In this case additional factors are required when complex damage presents at or very close to the break site and distorts the DNA structure such that NHEJ proteins cannot to process the ligation. Such damage must be removed by a nuclease and one of the main nuclease that has known to process the DNA ends is Artemis

As mentioned above, Artemis can trim 3′-PG terminated overhangs, as well as trim both DNA strands at blunt ends with 3′-PG or 3′-hydroxy termini (Povirk et al. 2007; Yannone et al. 2008). The rate of trimming is variable, and depends on the end structure. For example, the process is slower in trimming blunt ends than 5′ or 3′ overhangs (Povirk et al. 2007). Therefore, the function of Artemis may be to assist in the resolution of complex DSBs that are accompanied by damaged bases (Riballo et al. 2004). If so, it is important to determine the effect of base damage such as Tg on the stimulation of Artemis-mediated trimming or Tg will inhibit the Artemis binding and trimming of DNA ends as a first aim. As well as to determine to what extend the cell extract capable of ligating such ends without prior trimming as a second aim. Finally to test whether Tg will interfere with ligation during NHEJ and the interference depends on the distance of thymine glycol from the break ends. Here, we use substrates contain the most

commonly oxidized base, thymine glycol (Tg) at different positions; in cell extract assays and Artemis trimming assays. In order to measure the ability of cell extract in ligation of ends contain Tg and to ass the ability of Artemis to remove Tg at or near DNA ends, in the contest of cell extracts that contain all proteins needed to complete NHEJ.

II. Materials and Method

2.1 Substrate

Oligonucleotides 5′ATGCGGATCGCGTTGTC-Tg-3′ (Tg1), 5′ATGCGGATCGCGTTGC-Tg-C-3′(Tg2) and 5′-ATGCGGATCGCGTTG-Tg-CT-3′(Tg3) were obtained from Midland Certified Reagents (Midland, TX) and resuspended in TE (10 mM Tris–HCl, pH 8, and 0.1 mM EDTA). 50 pmoles of the oligonucleotide are labeled with 1mCi [γ-32P] ATP using Polynucleotide kinase (PNK) for 1 hr at 37 °C. Each oligomer was then annealed to an equal quantity of a complementary 5'- phosphate-containing, 3′ GTATACGCCTAGCGCAACAGAp-5′ or 3'GTATACGCCTAGCGCAACGAGp5', via heating to 80°C followed by slow cooling to 10°C. The duplexes generated by this annealing process included, on one end, a 3-base 3′ underhang that was complementary to the 5′ overhang created by BstAPI digestion of pUC19. The other end of the duplex was blunt.

2.2 Electroelution of the modified substrate

11 inches of pretreated dialysis tubing (6,000-8,000 molecular weight) was cut and tied at one end. The gel box was filled with buffer composed of 20mM Tris, pH8 and 1mM EDTA (the Elution buffer). The gel slice containing the desired DNA band was placed in the dialysis tubing and the other end of it was knotted to remove any air bubbles. The DNA was eluted overnight at 50 V. Then dialysis bag was cut and the buffer containing DNA substrate was collected into 15

ml centrifuge rubes. The solution was filtered through 0.45μm filters to remove any debris and was concentrated by micro- concentration to about 500 μl using centricon-100 (Amicon). The concentrate was collected into 1.5 ml tubes and precipitated with 1/9 volumes of NaOAc and 2.5 volumes of 100% EtOH, then washed with 70% EtOH and dissolved in 50 μl TE.

2.3 Polyacrylamide gel electrophoresis

Polyacrylamide gels (20x30x0.08cm) for electrophoretic separation contained 20% acrylamide: bisacrylamide in a ratio of 20:1 with urea added to a final concentration of 8.3 M for electrophoretic separations. After the urea dissolved, the mixture was cooled to room temperature before adding 0.075% (w/v) ammonium persulfate and 0.03% (v/v) TEMED (N', N',N',N'-tetramethylethylene diamine). The gel was allowed to polymerize for 1 hour. Samples then loaded into the wells of the gel and electrophoresed at constant power of 42 watts for 3-4 hours in 1X TEB running buffer.

2.4 Cleavage of pUC19 and ligation to oligomeric duplexes

17μg of the pUC19 DNA digested with 50 units of KasI (NEB) in 0.3 ml of NEB CutSmart buffer for 3 hrs at 37°C digested with 50 units of BstAPI in the same buffer for 3 hrs at 60°C. The 2.6-kb KasI-BstAPI fragment was purified on an 0.8% agrose gel, electroeluted and concentrated with an Amicon ultracel 100k centrifugal filter. To ligate the annealed labeled Tg-containing oligomers to the BstAPI digested end of pUC19, 10 pmole of annealed oligomers were combined with (~1.7 pmole) of KasI/BstAPI fragment and treated with 12,000 units (4 μl) T7 DNA ligase in

130µl of ligation buffer for 2 hrs at 25°C. Ligation products were extracted with phenol/chloroform and ethanol precipitated, then digested with SmaI (500 units) for 2 hours at 25°C in 50 µl CutSmart buffer. This removes 177 bp from the KasI-end and leaves a blunt end. The resulting 2.6-kb substrate bearing one Tg-modified end was electroeluted and concentrated as above. T7 ligase was used in place of T4 ligase because it preferentially ligates only cohesive and not blunt ends (Doherty at al. 1996; Subramanya et al.1996). Nevertheless, with the unmodified control oligomers, some self-ligation was detected and therefore for these oligomers the T7 ligase was reduced to only 2400 units. The ligation products were purified by agarose gel electrophoresis and Electroelution (Bennett et al. 1996). The linearized plasmid containing the 5′-ATGCGGATCGCGTTGTC-Tg-3′ sequence was referred to as the Tg1 substrate, while (the one containing the 5′-ATGCGGATCGCGTTG-Tg-CT-3′ sequence was referred to as the Tg3 substrate. The plasmid containing the 5′-ATGCGGATCGCGTTGTCT-3′ sequence was used as a control.

2.5 End joining assays using cell extracts

Reactions with whole–cell extracts contained 50 mM triethanolammonium acetate (pH8), 1 mM ATP, 1 mM dithiothreitol, 50 µg/ml BSA , 1.3 mM Mg(OAc)2 and dNTPs at 100 µM each. Typically a 16-µl reaction contained 10.4 µl of extract, resulting in a final concentration of 8 mg/ml protein. In some cases 100 nM recombinant XLF, 90 nM Artemis and or 100 mM ddTTP were added. Substrate (10-100 ng) was added and the reaction mixed by pipetting and incubated at 37 °C for 6 hrs. Samples were deproteinized by adding 1mg/ml of Proteinase K for 3 hrs at 56°C in order to stop reaction. DNA was then extracted with phenol/chloroform and precipitated

with ethanol for at least one hour. After the extraction and precipitation, all samples were dissolved in 32 µl of TE. Aliquots were treated with 10 unites of EndoIII in 2 µl 10X EndoIII buffer and incubated in 37°C for 2 hrs. The sample was then extracted with 20 µl of phenol/chloroform and precipitated with 45 µl of ethanol. All the samples were digested with 20 units of NdeI and PstI in (4 µl) of 10X CutSmart buffer (New England Biolabs) and incubated at 37°C for 3 hrs. Samples were subjected to electrophoresis in 20% polyacrylamide sequencing gels. Gels were exposed to phosphor imaging screen fro 2 days at -70°C. Data were analyzed using a Typhoon 9100 imager (GE Healthcare Bio-Sciences, Pittsburgh) and ImageQuant 3.1 or 5.1software (GE Healthcare Bio-Sciences, Pittsburgh).

2.6 Statistics

Error bars represent standard error of mean (SEM) for at least three independent experiments. Unpaired two-tailed t-tests were performed and the data were reported as significant for P values <0.05.

III. Results

3.1 Ligation of substrates with T4 DNA ligase to test interference by thymine glycol

The presence of thymine glycol at or near the termini of DNA double strand breaks can interfere with ligation efficiency, independent of the NHEJ process. Therefore, as the first step in this investigation, it was important to determine whether the substrates to be used in NHEJ-related assays could be ligated by T4 DNA ligase; an enzyme that joins DNA ends with either cohesive or blunt termini. Linearized substrate plasmids containing ^{32}P labeled oligonucleotide duplexes with either normal thymine or Tg at the termini (control and Tg1 plasmids, respectively), were evaluated for ligation with or without T4 DNA ligase. They were then digested with either NdeI, PstI, or both, in order to derive fragments that were diagnostic of the efficiency of ligation (Figure 3-1). In this assay, the unprocessed substrate is indicated by a 19-base labeled fragment released by NdeI. For Tg-containing substrate, this 19-mer forms a doublet due to different stereoisomers of Tg.

A fragment of NdeI-and PstI-digested DNA from a ligated plasmid would appear as a 44-bp band. In case of intermolecular ligation between two oligonucleotide ends, digestion with NdeI (with or without PstI) would yield a 36-bp fragment.

Assay results indicated that T4 ligase could ligate the control plasmid, but not the plasmid with Tg at the very end of the DSB (Figure 3-2). Ligation of the control plasmid with T4 ligase, followed by digestion with NdeI and PstI restriction enzymes, produced the expected 44-bp band

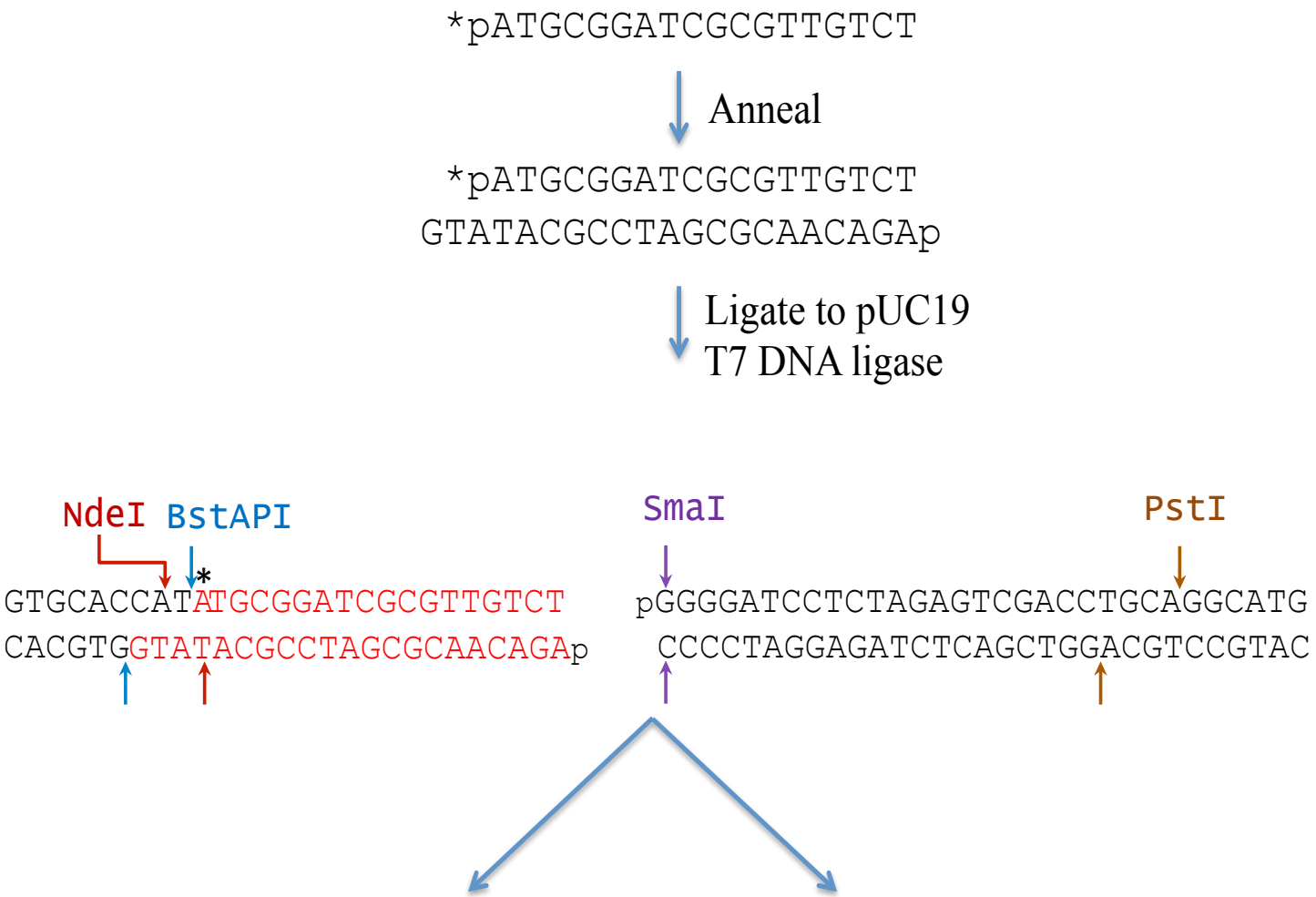
and the percent of ligation was (32%). This band was not seen in the lane corresponding to the analogous set of reactions performed on the Tg1 plasmid substrate. There was a detectable intermolecular ligation by T4 ligase, as indicted by presence of a 36-bp labeled fragment.

A similar assay was performed on plasmid containing a labeled oligomeric duplex with Tg located two bases from the break. Again, T4 ligase could ligate some of the control plasmid with normal thymine but in the case of Tg2, ligation did not occur. (Figure 3-3)

The assay was repeated with plasmid containing labeled oligomeric duplex with Tg located three bases from the break site (Tg3). T4 ligase could ligate plasmid with Tg3, but at very low efficiency (6.7%) compared to normal thymine (35%) (normal vs. Tg3 $p<0.005$), (Figure 3-4B). Taken together, these results indicate that the presence of Tg can completely inhibit ligation of DSBs when located at the first or second position from the terminus, but ligation is only partially inhibited if Tg occurs at the third base from DSB.

A

Method Summary



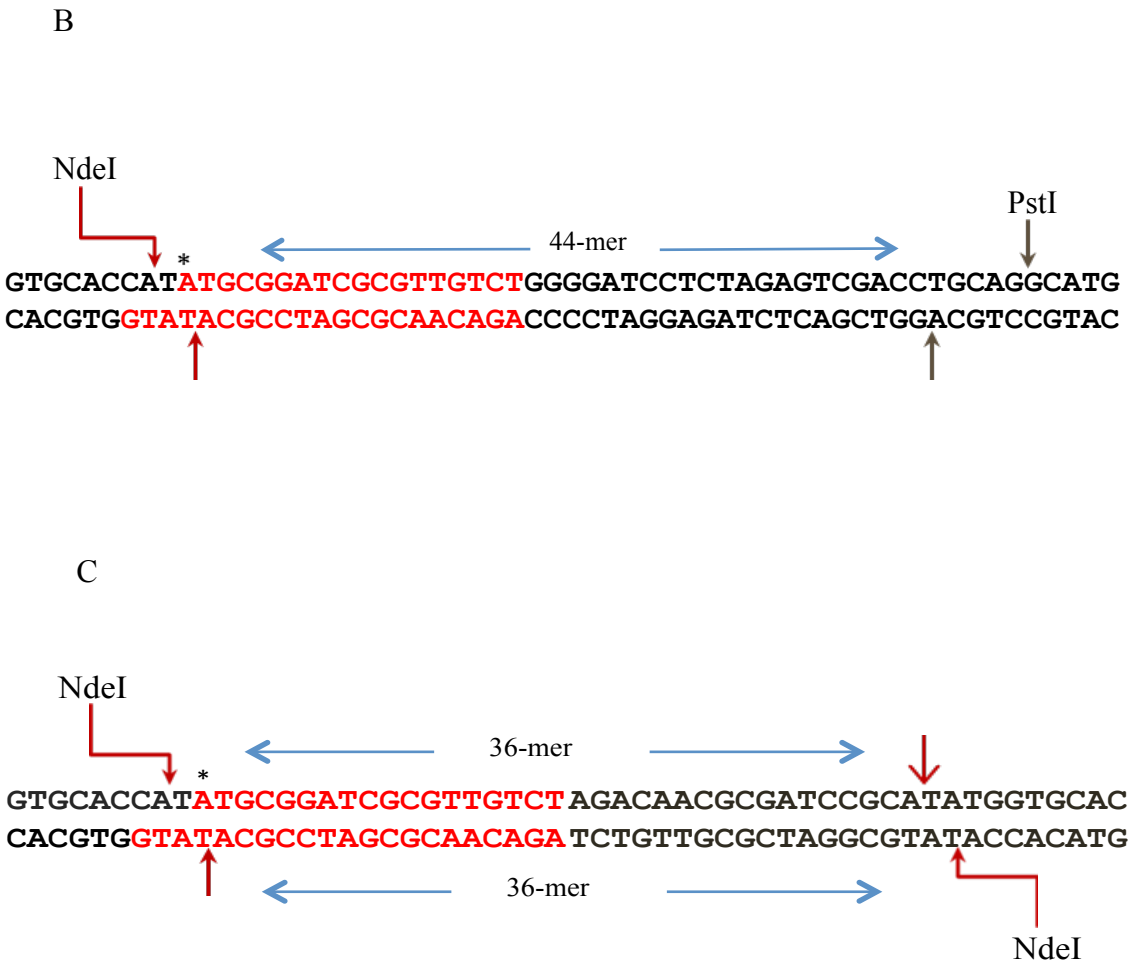


Figure 3-1. Schema of assay protocol. (A) Radioactively labeled oligomeric duplexes, containing normal thymine were ligated onto digested pUC19. Following attempted ligation with T4 DNA ligase or with cell extract containing DNA Ligase IV, or Artemis trimming assays. Plasmids were digested with either NdeI, PstI, or both. Fragment sizes on autoradiograph revealed the extent of ligation of the various substrates under the given conditions. T= thymine glycol location. (B) intramolecular ligation between the oligomeric duplex end and the end cut with SmaI, which generated a 44-nucleotide. (C) intermolecular ligation of two blunt ends of oligomeric duplex, which generated a 36-nucleotide fragment upon digestion with NdeI in both sides.

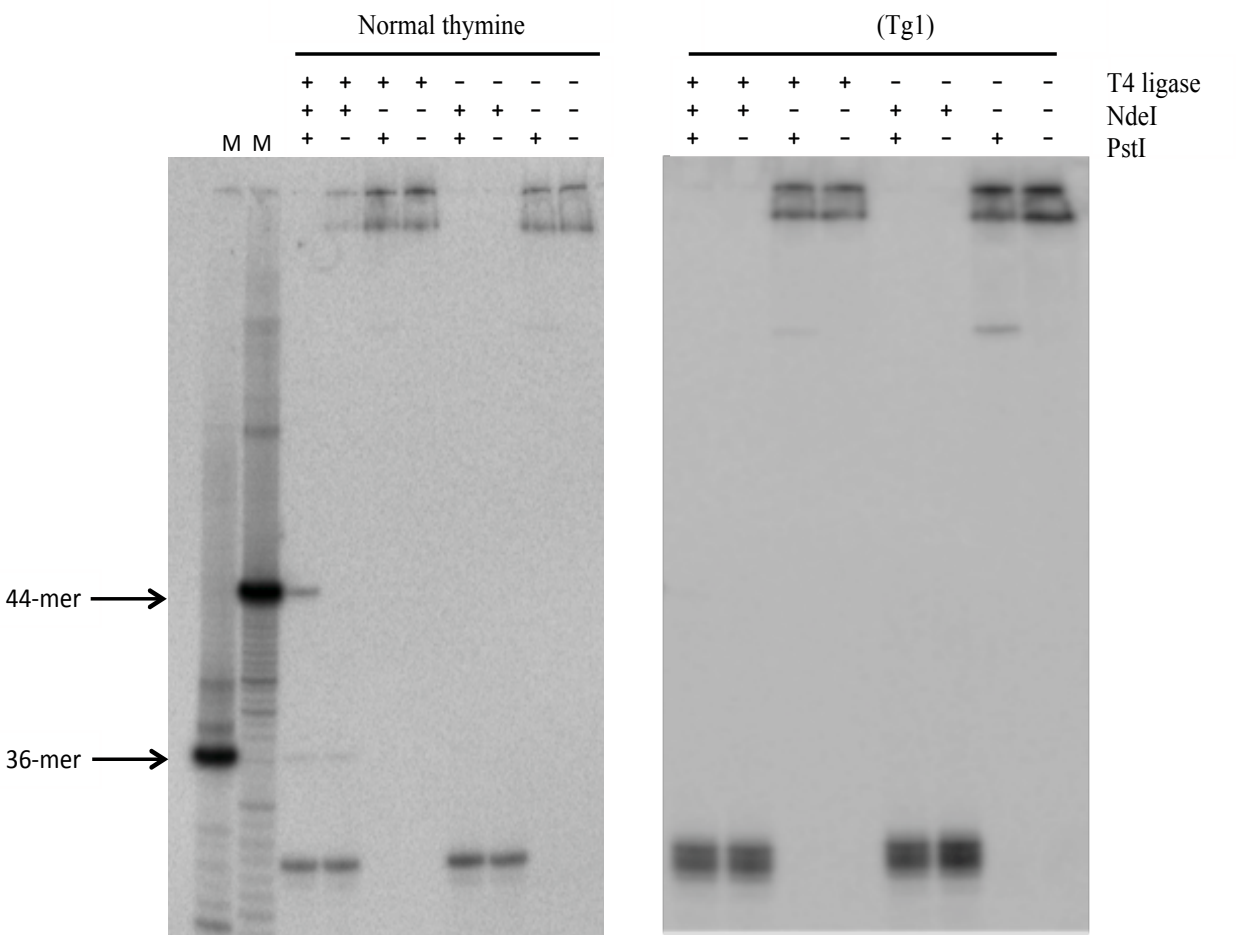


Figure 3-2. Ligation of (Tg1) with T4 DNA ligase followed by analytical restriction digest. M = marker lanes.

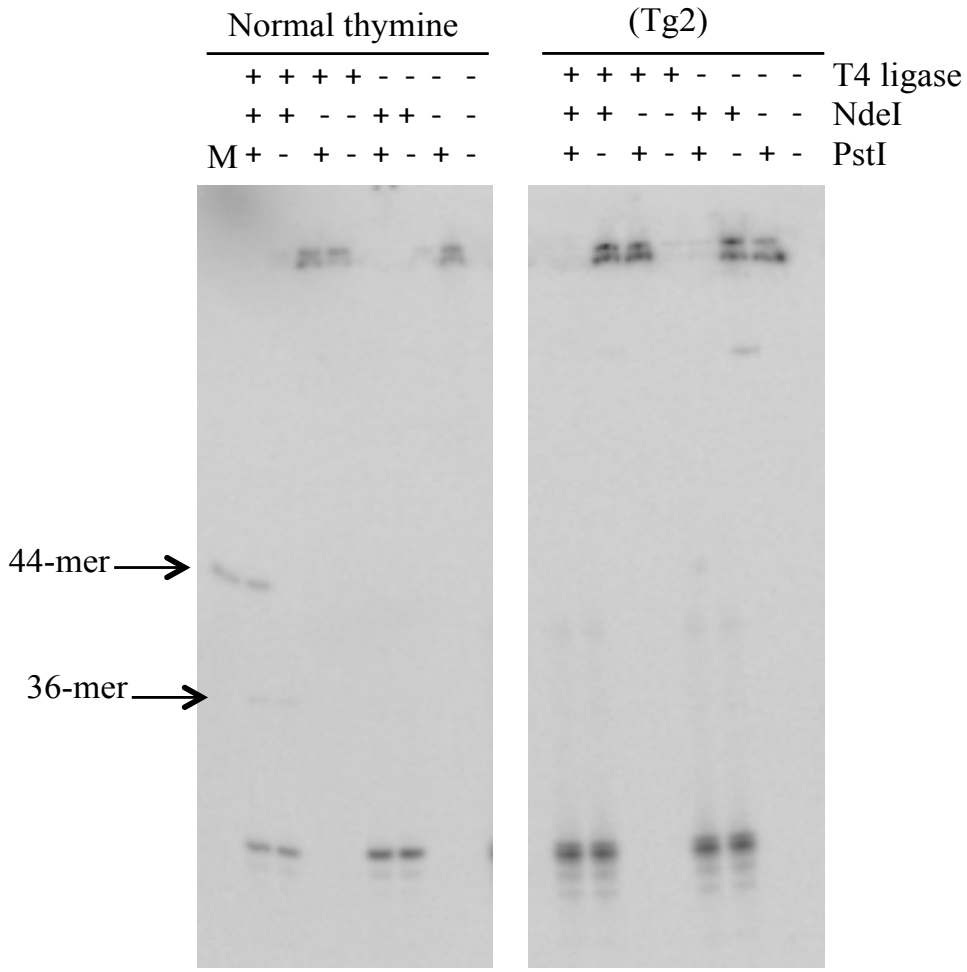


Figure 3-3. Ligation of (Tg2) with T4 DNA ligase followed by analytical restriction digest.
M = marker lanes.

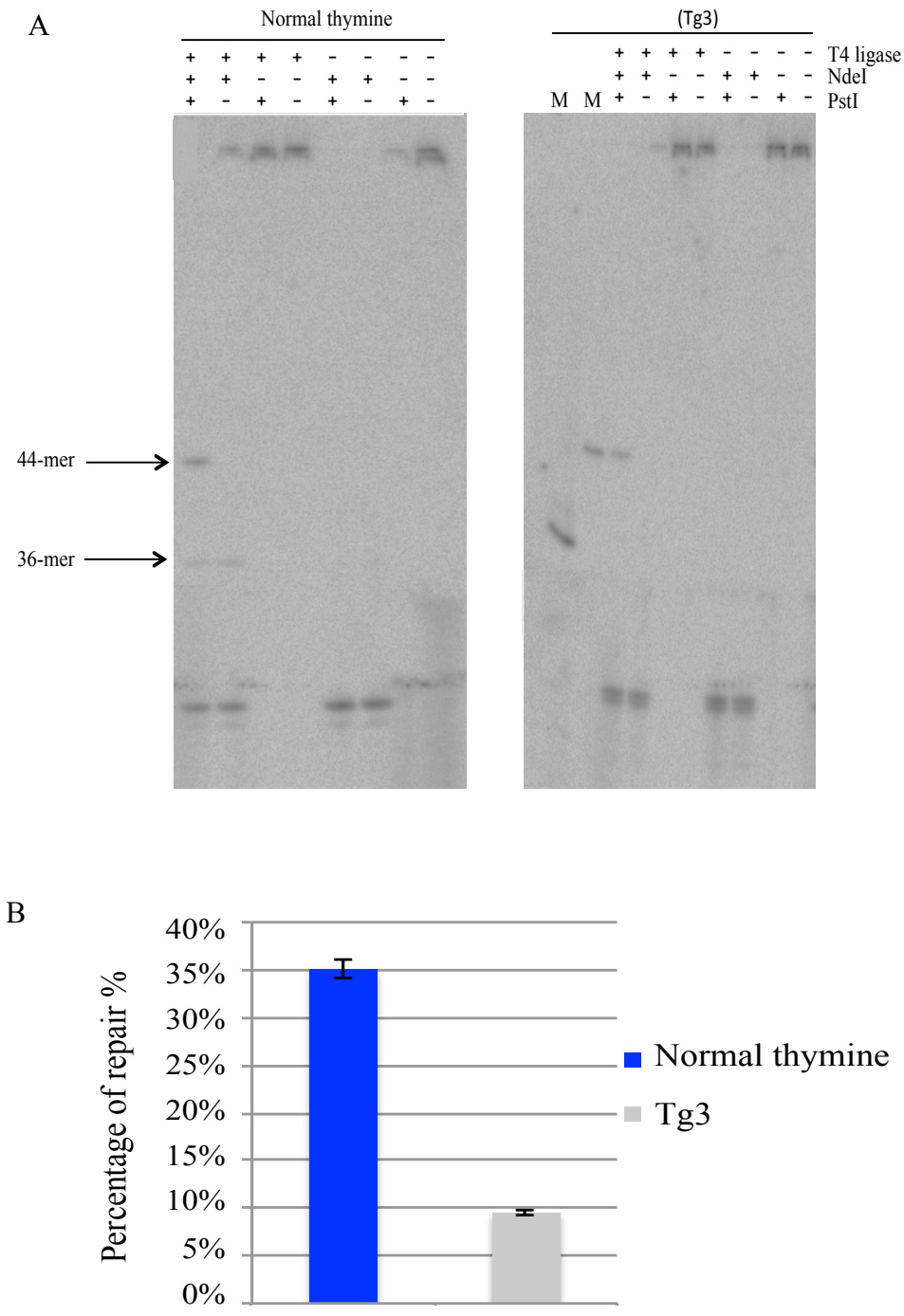


Figure 3-4. Ligation of (Tg3) with T4 DNA ligase followed by analytical restriction digest. M = marker lanes (B) Graph shows quantification percent of ligation of normal substrate vs. Tg3 in T4 DNA ligase.

3.2 Thymine glycol placed at termini blunt of DNA is poorly ligated.

To assess the ability of NHEJ to rejoin DSBs terminated by Tg lesions, a linearized plasmid with a thymine glycol introduced at the DSB terminus on one side (Tg1 plasmid), as well as a normal thymine control plasmid, was processed as substrates in a repair assay. Ligation of the plasmids was attempted using extracts from XLF deficient fibroblasts (BuS fibroblasts; Buck et al. 2009; Akopiants et al. 2009), with or without the addition of XLF. The extent of ligation was determined by NdeI and PstI digestion.

The data indicated that the presence of a terminal Tg severely hampered the ability of the cell extract to ligate the plasmids with terminal thymine glycols (1.83%), compared to the control plasmid with normal thymine (19.07%) (normal vs. Tg1 in cell extract assay with XLF,$p < 0.005$), and only allow for the a very little ligation (Figure 3-5). The control plasmid showed the 44-bp product indicting ligation between the oligomeric duplex end and the end cut with SmaI, Figure (3-5A). Ligation of two blunt ends of oligomeric duplex to generate a 36-nucleotide fragment upon digestion with NdeI was also observed (Figure 3-5B). In all cases end joining was completely dependent on XLF.

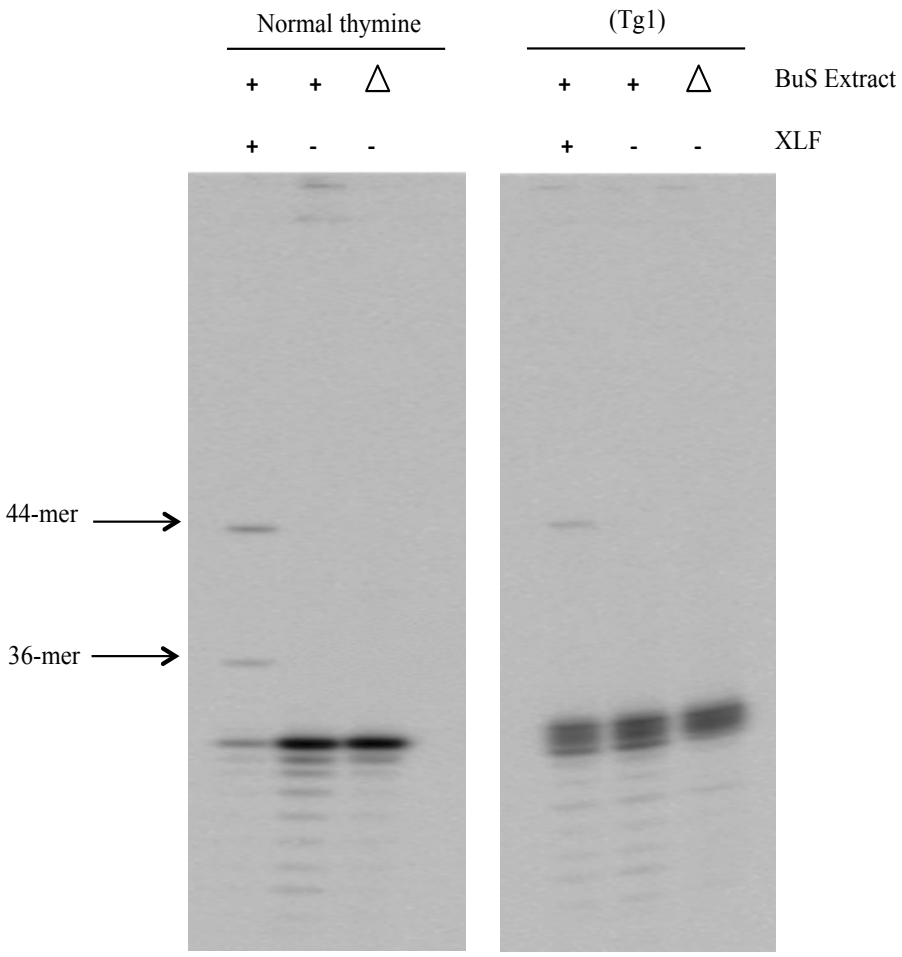


Figure 3-5. The ability of NHEJ to rejoin DSBs is affected by (Tg1). End joining assays, in whole-cell extracts of XLF-deficient BuS fibroblasts, plasmid with unmodified, normal thymine and plasmid with Tg at the terminus of the break site as substrate. Extracts were supplemented with 100nM XLF, as indicated. Δ = heat-inactivated extract.

3.3 Thymine glycol located at the second position from the terminus, allowed for some ligation.

Although the cell extracts were unable to ligate a linearized plasmid with a terminal Tg (Tg1 plasmid), it was possible that they would be able to ligate a linearized plasmid with a Tg near, but not at, the DSB (Tg2). A similar assay was therefore utilized to test this hypothesis, in which the (Tg2 plasmid) was added to extracts from XLF-deficient cells, with or without the addition of XLF, and the reaction products were digested with NdeI and PstI. The data indicated that cell extracts were be able to ligate very little of (Tg2 plasmid), with very low efficiency (0.06%) compared to control plasmid (19.07%), indicting that the ability of NHEJ to rejoin DSBs was severely impaired by the presence of Tg located at the second position (Tg2). Again ligation of either substrate occurred in whole-cell extracts of XLF-deficient BuS fibroblasts only with the addition of XLF, implicating classical NHEJ.

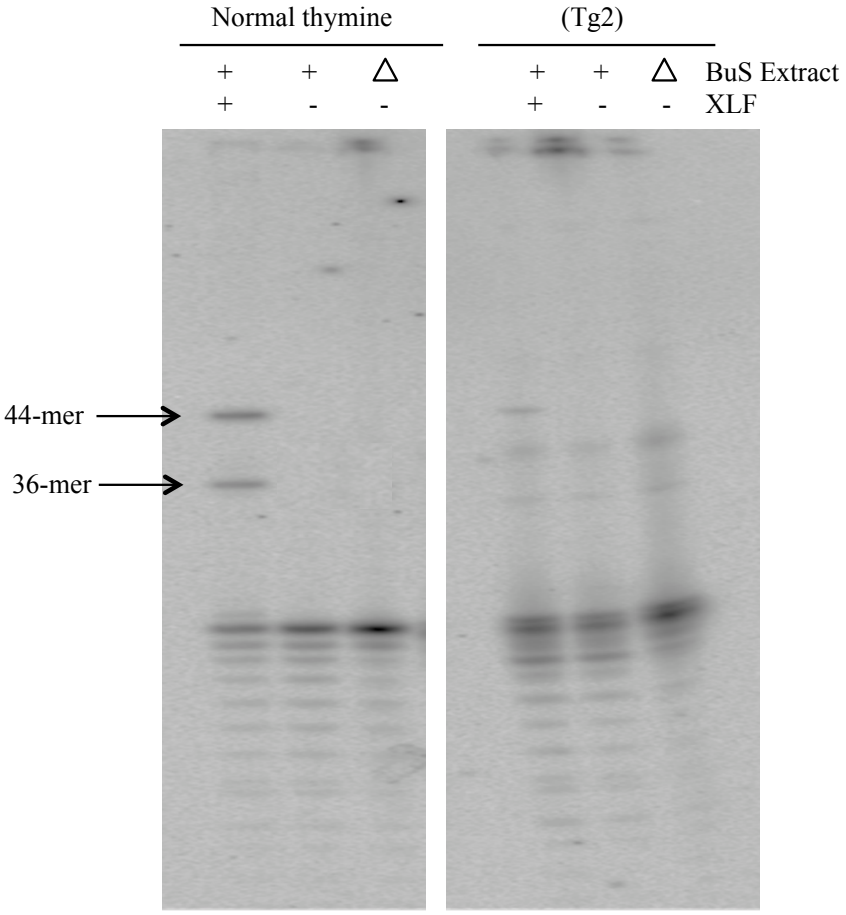


Figure 3-6. The ability of NHEJ to rejoin DSBs is affected by (Tg2). End joining assays, in whole-cell extracts of XLF-deficient BuS fibroblasts, plasmid with unmodified, normal thymine and plasmid with Tg at the second position of the break site. Extracts were supplemented with 100nM XLF, as indicated. Δ = heat-inactivated extract.

3.4 Thymine glycol placed at the third position from the blunt end allowed ligation.

Given that cell extracts were be able to ligate a plasmid with terminal Tg and plasmid with Tg located at the second position to break site with Tg located three bases from the break site. So again the reactions were performed with plasmids containing normal thymine and plasmid containing Tg3.

Cell extracts were able to ligate the Tg3 plasmid, as evidenced by the presence of the intramolecular 44-nt fragment and intermolecular 36-nt fragments. Thus, Tg three positions from the end did not inhibit the ligation of DNA termini (Figure 3-7).

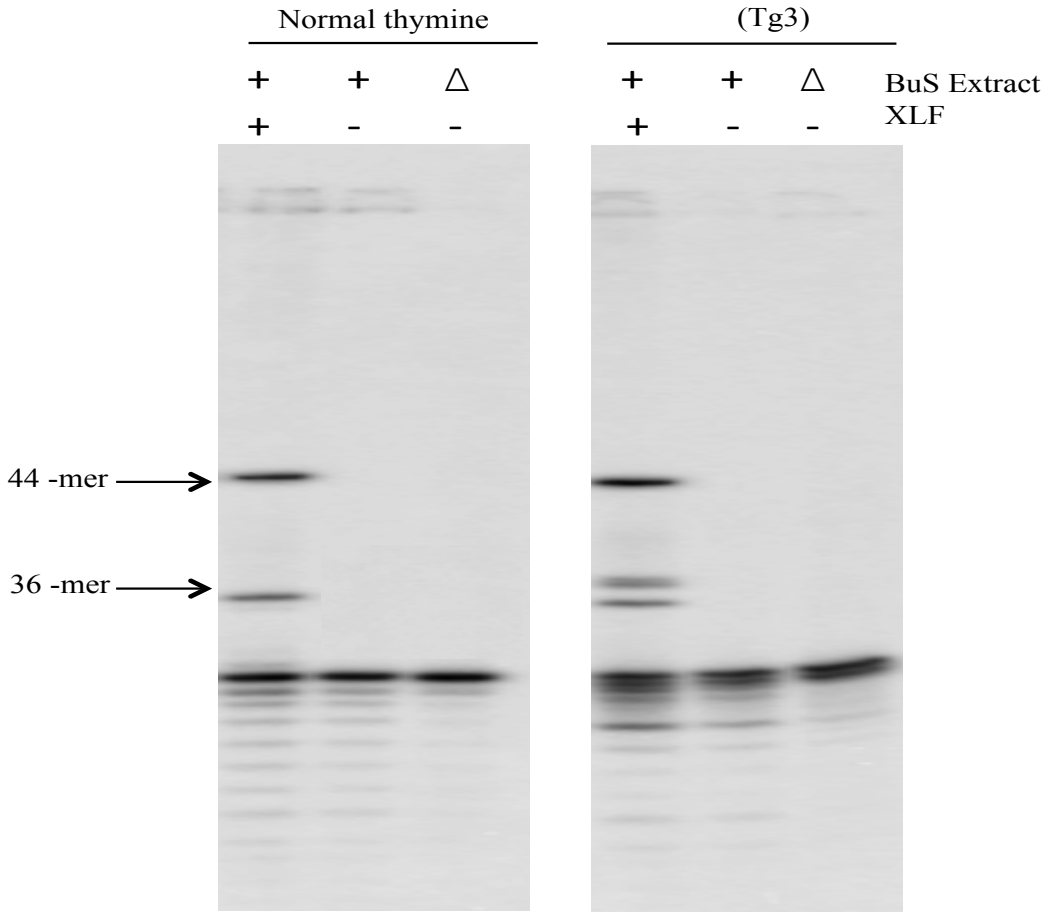


Figure 3-7. The ability of cell extract to rejoin DSBs is not inhibited by (Tg3). End joining assays, in whole-cell extracts of XLF-deficient BuS fibroblasts, plasmid with unmodified, normal thymine and plasmid with Tg at the third position of the break site. Extract were supplemented with 100nM XLF and/or EndoIII; Δ = heat-inactivated extract.

3.5 Effect of Artemis, Endonuclease III and ddTTP on end joining of linearized plasmids terminated with Tg

Artemis endonuclease has been proposed to trim damaged DNA ends that cannot otherwise be resolved. However, previous work (Mohapatra et al. 2013) indicates that Artemis concentrations in cell extracts are insufficient for trimming and that recombinant Artemis must be added to elicit significant trimming. In order to assess the role of Artemis in ligation of DSBs accompanied by modified nucleotide bases, such as Tg and to test whether the location of the offending base affects the endonuclease function of Artemis, the Artemis trimming assay was performed in the presence and the absence of XLF.

As already shown (Figure 3-2), ligation of Tg1 plasmid was observed in XLF-supplemented reactions, although the efficiency of ligation was exceedingly low (1.83%). Furthermore, Artemis was unable to substitute for XLF in the attempted ligation of the control plasmid (Figure 3-8 lane 9). As well as the reaction in the presence of XLF and Artemis did not affect the ligation versus XLF alone (Figure 3-8 lane 11). Moreover, although there was a small amount of end trimming in the extracts, as indicated by bands migrating faster than the substrate band, there was no increase in these bands with Artemis addition, indicating no detectable trimming by Artemis.

In addition, we wanted to determine whether the end joining occurred in the presence of Tg or only after removal of the Tg by nucleases. Therefore, in one reaction, the Tg1 plasmid following incubation in extracts and subsequent deproteinization was also treated with Endonuclease III (Endo III) from E. coli, which is known to remove damaged nucleotides with modified bases like Tg. Moreover in other reaction of the Tg1 plasmid with cell extracts, 2', 3'-Dideoxythymidine-

5'-Triphosphate (ddTTP) was added in place of dTTP. This compound is a sugar modified nucleoside triphosphate, where both 2' and 3' hydroxyl groups are absent, resulting in chain termination.

The inability of polymerases to extend from a dideoxy nucleotide causes the chain termination. So if any trimming of DNA ends occurred prior to the ligation of the control plasmid, with subsequent replacement of the thymidine at the end of the DSB, then substitution with dideoxythymidine would block the ligation, and the intensity of the bands representing the ligation products should be reduced. But no reduction in band intensity was observed (Figure 3-8B). indicating that the normal thymine control plasmid is ligated without the need for nucleases or polymerases. And the results indicted neither Endo III, nor ddTTP when added to the reactions containing added XLF had any effect on ligation, compared to addition of XLF alone.

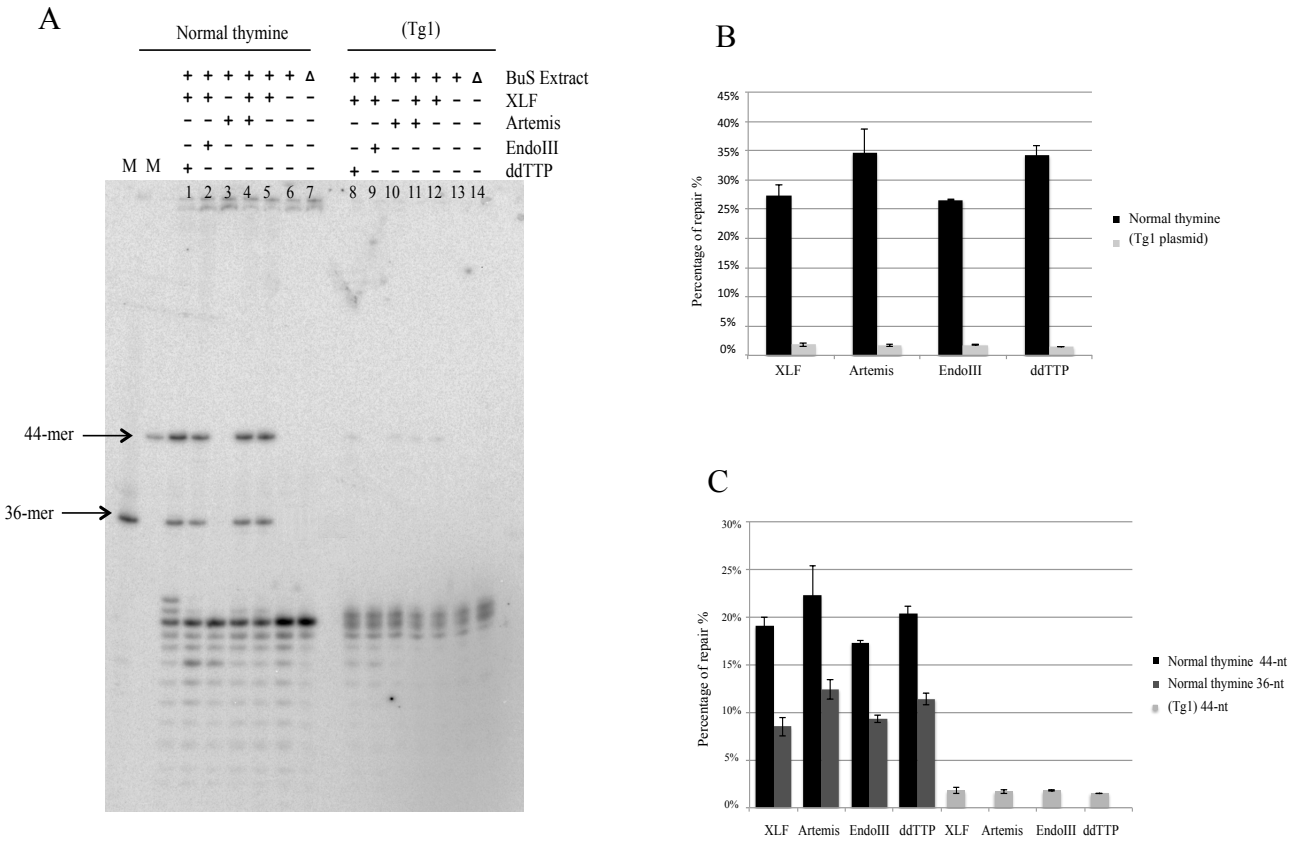


Figure 3-8. The effect of Artemis, Endo III and ddTTP on ligation efficiency of (Tg1). (A) Ligation of Tg1 plasmid in the presence of Artemis, EndoIII, or ddTTP to reactions containing added XLF had. (B) Graph shows quantification percent of repair for the sum of the 44-nt and 36-nt bands in the cell extract and Artemis assay with control plasmid and (Tg1) plasmid. (C) Graph shows quantification percent of repair for each band in the cell extract and Artemis assay with control plasmid and (Tg1) plasmid. Most lanes contain 5'- ^{32}P - labeled 44- and 36- base marker fragments of the sequence expected for blunt and ligation. Δ = heat-inactivated extract.

3.6 Effect of Artemis, Endonuclease III and ddTTP on end joining of linearized plasmid with Tg at the second position to DNA end

Since Artemis appeared to be unable to assist in the ligation of DSBs with terminal Tg modifications, the degree to which Artemis could excise Tg located a few nucleotides distant from DSB ends needed to be analyzed. The cell extract assays were repeated again, but with the control plasmid and Tg2 plasmid as template. Again, as above reaction, various combinations of XLF, Artemis, and ddTTP were added to XLF-deficient cell extracts. For Artemis reaction alone without the addition of XLF, Artemis was unable to substitute for XLF as far as rescuing ligation of Tg2 plasmids, as no 44- base end joining products were apparent in the presence of Artemis when XLF was absent. Addition of XLF resulted in a trace of 44- base product, suggesting a low level of ligation of the Tg2 substrate. Addition of ddTTP to the extracts reduced the formation of this product, suggesting that in some cases the Tg2 was removed and replaced by pol λ. However, subsequent treatment with EndoIII also decreased the 44-base product, suggesting that ligation sometimes occurred with Tg2 still present. In the presence of XLF, addition of Artemis appeared to generate a unique end-joining product slightly shorter than the predicted 44-mer, suggesting ligation of trimmed ends although the extent of joining was still very low. There was no detectable 36-base product of Tg2 under any condition, suggesting that presence of Tg at the second position of both DSB ends was an absolute block to end joining.

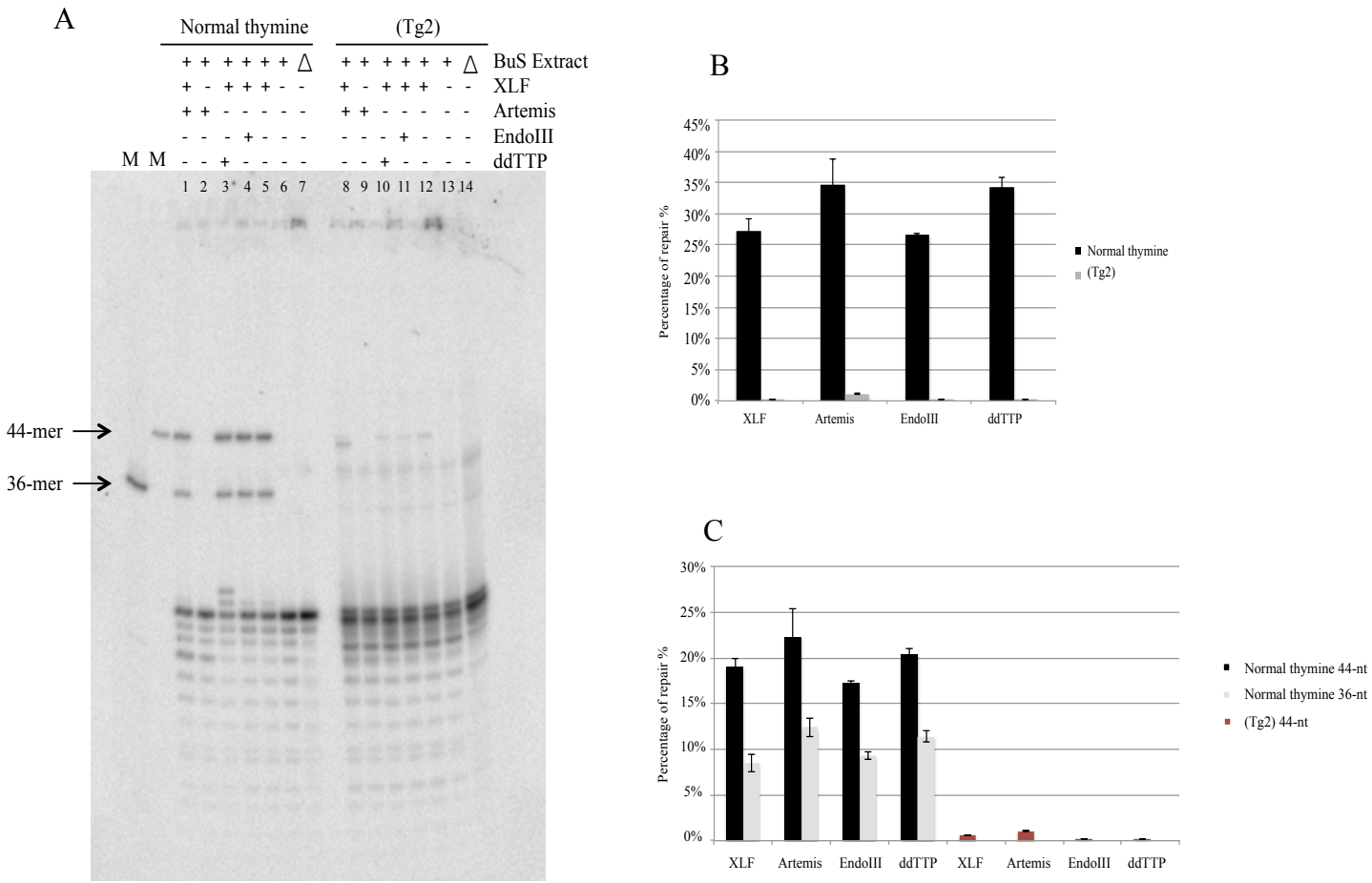


Figure 3-9. The effect of Artemis, EndoIII and ddTTP on ligation efficiency of (Tg2). (A) Ligation of Tg2 plasmid in the presence of Artemis, EndoIII, or ddTTP to reactions containing added XLF had. (B) Graph shows quantification percent of repair for the sum of the 44-nt and 36-nt bands in the cell extract and Artemis assay with control plasmid and (Tg2) plasmid. (C) Graph shows quantification percent of repair for each band in the cell extract and Artemis assay with control plasmid and (Tg2) plasmid. Most lanes contain 5'- ^{32}P - labeled 44- and 36- base marker fragments of the sequence expected for blunt and ligation. Δ = heat-inactivated extract.

3.7 Effect of Artemis, Endonuclease III and ddTTP on end joining of linearized plasmid with thymine glycol at the third position to DNA end.

The cell extract assays were also repeated, but with the control plasmid or Tg3 plasmid as substrate. Artemis was unable to substitute for XLF as far as rescuing ligation of either control or Tg3 plasmids, and none of the additives (aside from XLF) had any appreciable effect on control plasmid ligation. However, while overall ligation of the Tg3 plasmid appeared to be unaffected by Artemis, Endo III, or ddTTP, removal of Tg was affected.

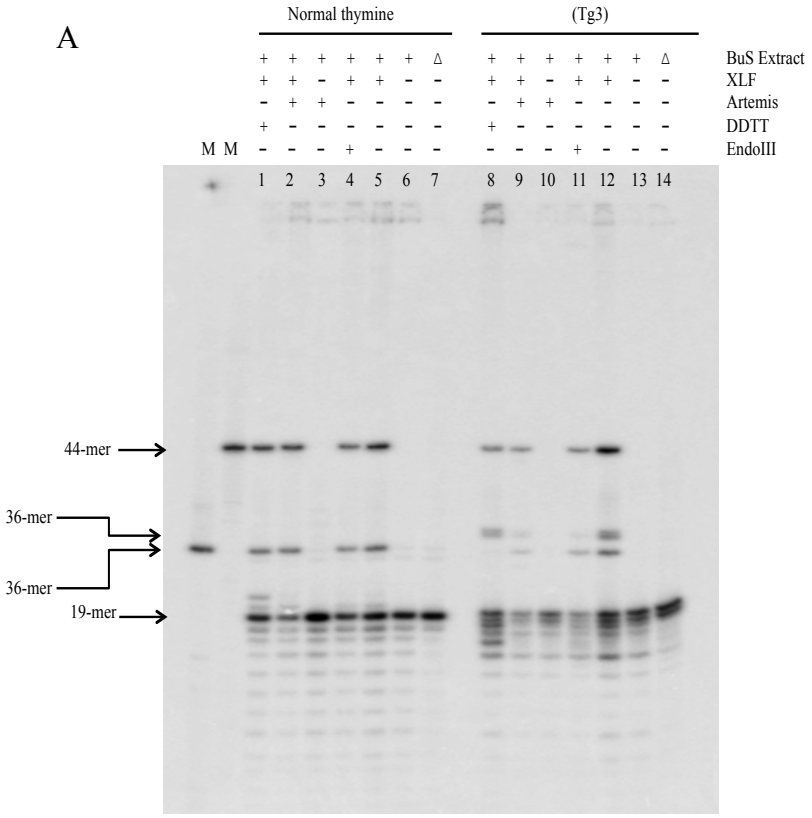
Compared to XLF alone, addition of XLF plus Artemis seemed to cause a shift in the ratio of the intensities of the bands of the 36 duplicate 3.1% of upper band and 3.5% for the bottom band compared to 36 duplicate 6.03% of the upper band and 5.6% to the lower band of XLF alone (Figure 3-10C). This result indicates that Artemis could remove Tg modifications located near the DSB end, but with low efficiency.

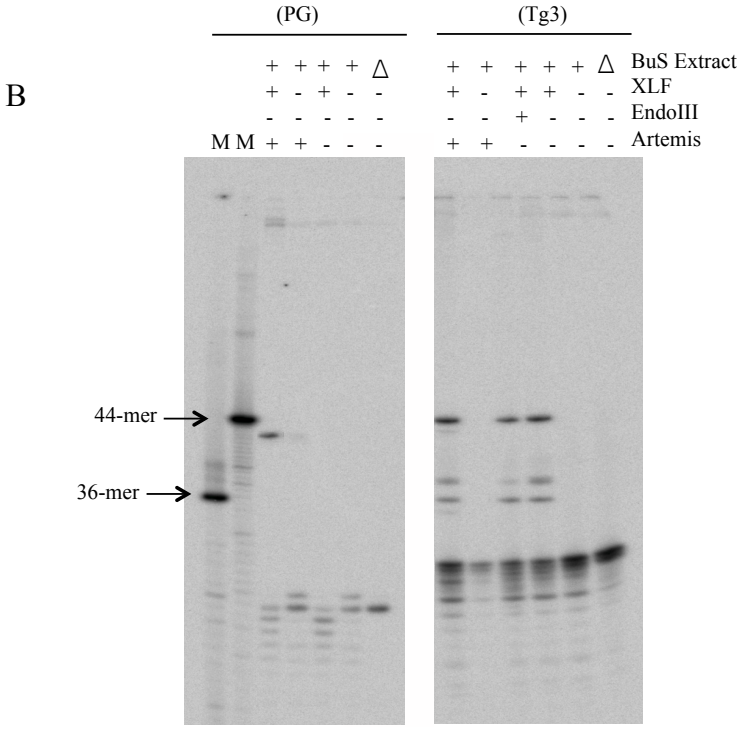
Addition of Endonuclease III and XLF resulted in a much more significant shift in intensities, such that the of 36-bp doublet was definitely less intense (1.6%), compared to XLF alone (6.03%) of Tg3. This result suggests that Endonuclease III could cleave Tg that are still present in the ligated product, which leads to disappearance of the upper band of the doublet. In contrast, the lower band of the doublet showed little or no change (5.1% compared to 5.6% with XLF alone), which indicates that the lower band did not contain thymine glycol consistent with its authentic unmodified 36-base marker (Figure 3-10). Thus, the disappearance of the upper band after addition of endonuclease III confirmed, again, that intermolecular ligation could occur without removal of thymine glycol near the end of the DSB.

The disappearance of the lower band of the 36-nt doublet after ligation and restriction enzyme digest in the reaction with ddTTP was presumably due to removal of the Tg (by endogenous

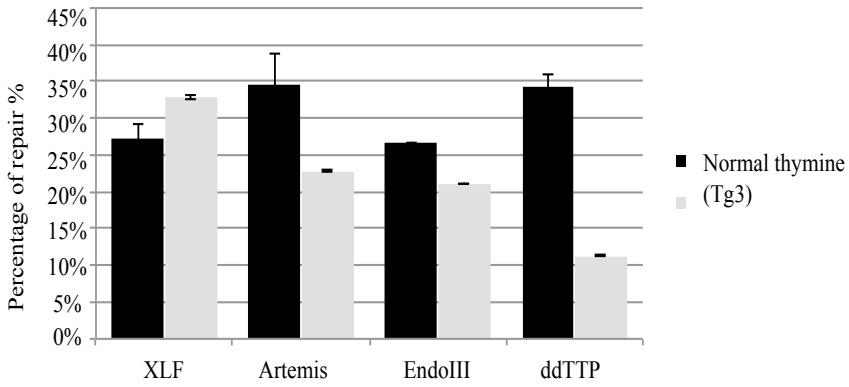
nucleases), followed by replacement with deoxythymidine by endogenous polymerases. The addition of ddTTP to the reaction caused the polymerases to fill in (ddTTP) in place of (dTTP), and the lack of a 3′ hydroxyl group prevented ligation. Thus, addition of ddTTP prevented the ligation of plasmids that had been processed by nucleases and polymerases in the cell extract, resulting in the disappearance of the lower band of the doublet. As expected the upper band of the doublet was not affected by ddTTP addition, confirming that its formation did not involve Tg removal and filling, which is consistent with the conclusion that it was ligated with Tg still present.

Either addition of ddTTP or subsequent treatment with Endonuclease III also reduces the yield of 44-base end joining product, from (21.1%) to (6.4%) and (14%) respectively (Figure 3-10A, lane 8&11 versus lane12). Partial cleavage by EndoIII indicates that ligation of this product likewise sometimes occurred with Tg still present, while the decrease see with ddTTP suggests that Tg was sometimes replaced, just as with the 36- base product. It is unclear however, whether Tg removal occurred before or after ligation. If, as proposed, the residual 44-base product formed in the presence of ddTTP represents events where in the Tg remained present while the residual product detected after EndoIII cleavage represents events wherein Tg was replaced, then the sum of the two should be approximately equal to the level of 44-base product seen without either ddTTP or EndoIII, as is observed (Figure 3-10C). The apparent alteration of fragment mobility induced by Tg in the 36-base but not the 44-base product is probably due to the palindromic sequence of the 36-base product (see Figure3-10A). Even under denaturing gel conditions, the palindrome could renature at least transiently into a double – strand hairpin, and Tg would likely interfere with that renaturation.


A
Normal thymine
(Tg3)
BuS Extract
XLF
Artemis
DDTT
EndoIII
M M
1 2 3 4 5 6 7 8 9 10 11 12 13 14
44-mer
36-mer
36-mer
19-mer


B
(PG)
(Tg3)
BuS Extract
XLF
EndoIII
Artemis
M M
44-mer
36-mer

C



D

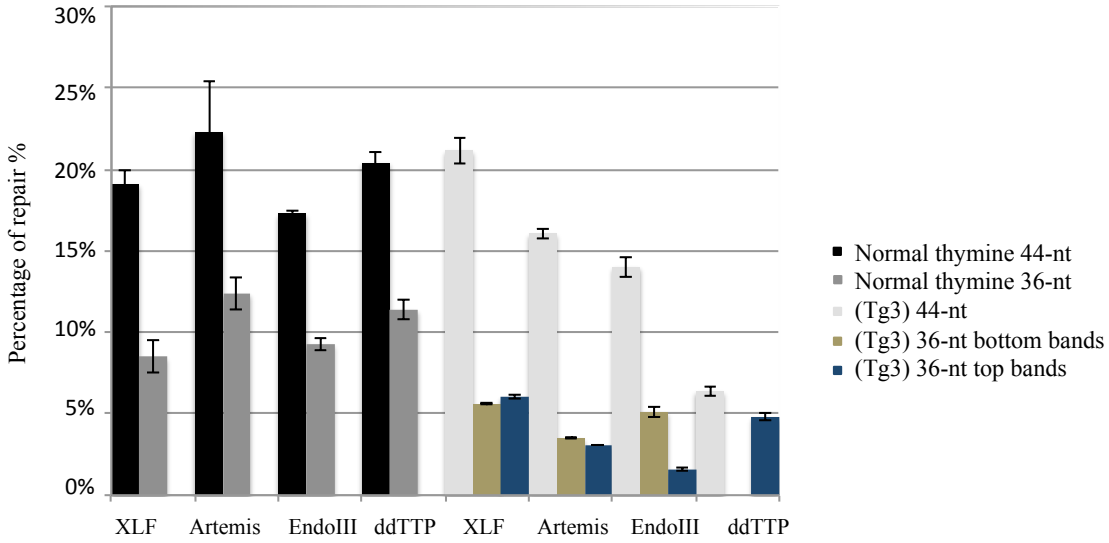


Figure 3-10. The effect of Artemis, EndoIII and ddTTP on ligation efficiency of (Tg3). (A) Ligation of Tg3 plasmid in the presence of Artemis, EndoIII, or ddTTP to reactions containing added XLF had. (B) a positive control contains a different sequence with PG on a 5-base 3'-overhange, a substrate previously shown to be efficiently trimmed by Artemis, and subsequently ligated in Artemis supplemented extracts (C) Graph shows quantification percent of repair for the sum of the 44-nt and 36-nt bands in the cell extract and Artemis assay with control plasmid and (Tg3) plasmid. (D) Graph shows quantification percent of repair for each band in the cell extract and Artemis assay with control plasmid and (Tg3) plasmid. Most lanes contain 5'- ^{32}P - labeled 44- and 36- base marker fragments of the sequence expected for blunt and ligation. Δ = heat-inactivated extract.

IV. Discussion

Artemis is one of the major nucleases that process DNA ends as part of the NHEJ pathway (Mohapatra et al. 2011; Beck et al. 2011; Riballo et al. 2004; Lee et al. 2005). The core NHEJ repair pathway proteins, comprising of DNA-PK, XLF, the X4L4 complex, and a subset of polymerases, has the ability to reunite and ligate termini of DNA breaks that do not require extensive processing in order to join the termini with high efficiently (Budman J et al., 2005; Guirouilh–Barbat J et al., 2004). However, many DSB are associated with complex lesions that block access to the DNA termini, and that can be barriers to the end joining step of the pathway (Zhou et al. 2008; Datta et al. 2001; Zhou et al. 2005; Pastwa et al. 2003). Thus, we wanted to assess the effect of the damaged base Tg on the ligation and whether the DNA end-trimming activities of Artemis can generate and undamaged end that is suitable for patching and ligation by removing blocked termini and damaged bases, either at or near the termini of blunt ends.
My work examines the end joining activities of cell extracts on normal thymine control plasmid or a Tg-containing plasmid, with or without XLF, Artemis, EndoIII, or ddTTP. Whether the substrate is normal control plasmid, Tg1, Tg2 orTg3 plasmid, no ligations occur without XLF.
In the reactions involving the normal thymine control plasmid, as long as we add XLF to the cell extract, regardless of whether or not we add Artemis or EndoIII or ddTTP, we consistently detect two ligation products. First, the intramolecular or head-to-tail ligation of the oligomer duplex end to the SmaI end, which results in the 44-nt fragment after PstI and NdeI digestion.

Second, the intermolecular ligation of the oligomeric duplex ends of two plasmids, resulting in the 36-nt fragment band after NdeI digestion. Since there is no Tg in the normal thymine control plasmid, addition of endonuclease III has no effect. Similarly, since the plasmid is ligated without any need for nucleases in the cell extract to remove any modified nucleotides, there is no need to replace any nucleotides and no need to fill in missing thymidines, neither normal thymidine (dTTP) nor ddTTP is used to fill in anything, and the ligation only requires a ligase.

In the Tg1 plasmid reactions with XLF, addition of endonuclease III or ddTTP has no effect (normal vs. Tg1 in cell extract assay with EndoIII, $P < 0.005$, with ddTTP, $p < 0.005$), (Figure 3-8B). EndonucleaseIII only works on dsDNA, so for endonuclease III to function on the Tg containing strand it may first require the complementary strand to be ligated. However, the presence of Tg introduces distortions in the DNA structure. Unlike the normal nucleotides, which stack one above the other, forming the "stairs" of the DNA "double-helix staircase" Tg does not stack, and is "extrahelical", in that it is twisted outside of the double-helix (Kao et al. 1993). The presence of Tg on one DNA strand also affects the complementary strand, by affecting the adenine base opposite to Tg to be distorted and extrahelical (Kao et al. 1993).

Since the adenine in the complementary strand of the Tg1 plasmid is at the very end of the DSB, it is right next to the position on the DNA backbone to which the DNA ligase needs to bind in order for the complementary strand to be ligated. However, because that adenine is extrahelical, it therefore is probably too distorted for the ligase to bind.

However this is not the case with Tg2, the addition of EndoIII or ddTTP has some effect by reducing the intensity of 44-nt fragments. Apparently, Tg when located at the second position has less distortion effect that Tg located at the terminal and adenine opposite to the Tg might not

get in the way of the binding of the ligase to the complementary strand which allow endonuclease III to hold onto the complementary strand cleave the Tg and that leads to reduce the intensity of the 44 fragment (Figure 3-9B) and it seems the same case with endogenous nucleases that were able to cleave Tg and the polymerase fill in with ddTTP and leads to reduction of the intensity of the 44 fragment (Figure 3-9B).

Previous work has demonstrated that Artemis can trim off 3′ overhangs with phosphoglycolate (PG) modifications (Povirk et al. 2007) with high efficiency and remove a PG modified nucleotides located at the terminus of a blunt ended DSB, although more slowly than a PG containing overhang (O'Driscoll and Jeggo, 2006). Moreover, in the presence of the full set of NHEJ proteins, Artemis interacts with DNA-PK to trim off the damaged DSB overhang end, producing ends that are suitable for patching and ligation. Some nucleases may display activity in resolving DSB ends in vitro, but they may not always participate in the repair of complex DSBs in vivo. Additional base damage at a complex DSB may interfere with access to the DSB ends, or somehow prevent the required interaction between the nuclease and other NHEJ proteins like Ku or PNA-PKcs. In fact, results of the present study show that nucleotides with modifications of thymine to Tg, if located at the very terminus of the DNA blunt end, do inhibit Artemis-mediated trimming and subsequent ligation. The ligation efficiency of the cell extract and XLF, with or without Artemis, is very low for the Tg1 plasmid, so we only get a faint band at 44-nt, and no band at 36-nt (normal 44 bands vs. Tg1 44-bands with Artmis, $p < 0.005$). This is not that surprising, since the presence of thymine glycol not only gives rise to a distortion in the strand in which is it located, but also causes a distortion in the adenine base of the nucleotide directly opposite, in the complementary strand (Kao et al. 1993). Likewise, Artemis can trim nucleotides from the 3′ end at the site of a complex DSB in order to remove modified bases, but

at a blunt end, must first trim nucleotides from the 5′ end of the complementary strand in order to create a 3′ overhang (Yannone et al. 2008). Therefore, the distortion of the adenine opposite to a Tg at a DSB likely interferes with the function of Artemis if the Tg is at the very end of the DSB. (Figure 3-8). However, apparently Artemis could trim some of Tg2 which was apparent from the shifting of the 44-nt band to a slightly to a shorter repair products this result could means that Tg, if located at the second base not at the break, could have the ability to stimulate the endonuclease Artemis to trim the substrate, but still that did not significantly affect the overall level of end joining ligation (Figure 3-9). With the thymine glycol located at the third positions to DSB, a higher frequency of intramolecular reactions can occur, since the thymidine glycol at that position does not interfere as much in the ligation process, compared to the Tg1 or Tg2 plasmids, and NdeI digest after such ligations produces a significant amount of 36-nt product. In the Tg3 gel, a doublet is visible in the 36 nt band region of the gel, as long as XLF is present in the reaction, whether or not Artemis or endonuclease III is added to the reaction. This indicates that, even in the reaction with just cell extract and XLF, DNA ligase IV could ligate the two ends while Tg is still there.

When the end joining products of Tg3 were treated with endonuclease III, we can see that the upper band of the 36-base doublet has partially disappeared (Figure3-10A, lane 11). In this case, the endonuclease III seems to be able to hold on to the complementary strand while removing the Tg from one strand. There are two nucleotides between the adenosine opposite the Tg and the break in the complementary strand, so endonuclease III can bind to these while removing the Tg. Moreover, while the adenine in the complementary strand is still structurally distorted because it is opposite to the Tg, it is not the last nucleotide of the DSB, so it does not interfere with the binding of ligases at the DSB end. It is likely that Ligase IV can ligate the break in the

complementary strand of the Tg3 plasmid, giving endonuclease III something to hold on to while removing the Tg. As a result, endonuclease III can remove the Tg and cleave the product, which decreases the intensity of the upper band of the 36nt doublet. Also after quantifying the upper and the lower bands of Tg3 with added Artemis it appears that Artemis could reduce the intensity of the 36 doublet band to (6.6%) compared to (12.2%) with XLF alone and (Figure3-10 B).

This result suggests that Tg located at the third position from the DSB could stimulate the trimming activity of Artemis and it could have access into the strand even with distortion produced by adenine opposite to Tg3 could trim Tg3, but the trimming did not lead to any increase in the overall ligation. Moreover, the endogenous nucleases may have been stimulated by Tg to access to the double strand to remove Tg and replacing it with ddTTP which leads to terminate the reaction because of the absence of a 3′ OH. Such events are represented by the 36 nt lower band of the doublet which disappears when ddTTP is added (Figure3-10 A, lane 8), because the plasmids which have been processed to remove Tg now contain ddTTP instead, and cannot be ligated at all. Therefore, the only band we can see for the ddTTP containing reaction is the product of ligation without removal of the Tg, which is the upper band of the doublet (Figure3-10 A, lane 8). On the other hand, it is also possible that Tg removal and replacement with ddTTP occurred after the ends were already ligated, which would likewise result in loss of the lower band of the doublet.

V.Conclusions

Extracts from XLF-deficient cells, in which the core NHEJ pathway was reconstituted by the addition of recombinant XLF, but which were not enriched for Artemis, could ligate the two ends of a complex DSB with thymine glycol located at the third base to the break site with high efficiency but ligation seems to be severely inhibited by the presence of thymine glycol at the terminal or at the second position to DSB. Thus, most DSBs with nearby Tg will likely be directly ligated by cell extract despite presence of Tg but Tg at the extreme end or located next to the last base of a DSB could be a major barrier to repair. Furthermore, nucleotides with modifications of thymine to Tg1 show no susceptibility Artemis-mediated trimming and subsequent ligation whereas Tg2 and Tg3 appear to allow Artemis mediated trimming to a limited extent.

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